

# ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ  
ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

## ΠΡΩΤΕΩΜΙΚΗ ΜΕΛΕΤΗ ΤΗΣ ΜΑΣΤΙΤΙΔΑΣ ΑΠΟ *MANNHEIMIA HAEMOLYTICA* ΣΕ ΠΡΟΒΑΤΑ

**ΑΓΓΕΛΙΚΗ Η. ΚΑΤΣΑΦΑΔΟΥ**

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### ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

που εκπονήθηκε στην Κλινική Μαιευτικής και Αναπαραγωγής  
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*Remember to look up at the stars and not down at your feet.  
Try to make sense of what you see,  
and wonder about what makes the universe exist.  
Be curious.  
And however difficult life may seem,  
there is always something you can do and succeed at.  
It matters that you don't just give up.*

S.W. Hawking

**Η διατριβή είναι αφιερωμένη στην οικογένειά μου**

# ΠΕΡΙΛΗΨΗ

Η παρούσα διατριβή εστιάζεται στην πρωτεωμική μελέτη της πειραματικής μαστίτιδας από *Mannheimia haemolytica* σε πρόβατα, χρησιμοποιώντας καθιερωμένα πρότυπα πειραματικής μόλυνσης, και αποσκοπεί ειδικότερα: (α) στη μελέτη της διαφορικής έκφρασης πρωτεϊνών επακόλουθα της πρόκλησης μαστίτιδας από *M. haemolytica* σε προβατίνες και (β) στην αξιολόγηση της σημασίας της καθελυσιδίνης-1 στο γάλα των προβατίνων ως διαγνωστικό μέσο για την ανίχνευση της υποκλινικής μαστίτιδας.

Η διατριβή χωρίζεται σε τρία κεφάλαια και ακολουθεί η Γενική Συζήτηση.

Στο Κεφάλαιο I, ανασκοπείται η σχετική βιβλιογραφία. Το Κεφάλαιο υποδιαιρείται σε δύο τμήματα. Στο τμήμα A, ανασκοπείται συνοπτικά η βιβλιογραφία σχετικά με την πρωτεωμική τεχνολογία και τις μεθοδολογίες που ακολουθούνται. Στο τμήμα B ανασκοπείται η βιβλιογραφία η σχετική με την εφαρμογή της πρωτεωμικής στην κτηνιατρική επιστήμη.

Στο Κεφάλαιο II, μετά από σύντομη ανασκόπηση για το βακτήριο *M. haemolytica* και το ρόλο του στη μαστίτιδα (τμήμα A), περιγράφεται ένας πειραματισμός, στον οποίο πραγματοποιήθηκε πρωτεωμική ανάλυση του αίματος και του γάλακτος προβατίνων έπειτα από ενοφθαλμισμό του μαστού με *M. haemolytica* (τμήματα B και Γ).

Προκλήθηκε μαστίτιδα σε προβατίνες (n=5) στη γαλακτική περίοδο με ενοφθαλμισμό ενός στελέχους *M. haemolytica* με γνωστή παθογόνο δράση εντός του θηλαίου πόρου του μαστού, η δε ετερόπλευρη πλευρά του μαστού χρησιμοποιήθηκε ως μάρτυρας. Για επιβεβαίωση της εκδήλωσης μαστίτιδας και για παρακολούθηση της εξέλιξης της νόσου, χρησιμοποιήθηκαν καθιερωμένες κλινικές, μικροβιολογικές, κυτταρολογικές και ιστοπαθολογικές τεχνικές. Δείγματα αίματος (για εξαγωγή πλάσματος) και γάλακτος (για εξαγωγή ορού γάλακτος) συλλέχθηκαν διαδοχικά πριν από και μετά τον ενοφθαλμισμό, συνολικά σε 6 ή 7 χρονικά σημεία ανά προβατίνα μέχρι την 4η ημέρα μετά τον ενοφθαλμισμό. Για την πρωτεωμική ανάλυση, οι πρωτεΐνες διαχωρίστηκαν με δισδιάστατη ηλεκτροφόρηση πηκτώματος πολυακριλαμιδίου σε όλα τα δείγματα και στα πηκτώματα ανιχνεύθηκαν οι διαφορικά εκφρασμένες πρωτεΐνες. Επιλέχθηκαν οι κηλίδες πρωτεϊνών στα πηκτώματα και οι πρωτεΐνες ταυτοποιήθηκαν με φασματομετρία μάζας, χρησιμοποιώντας ιοντισμό-εκρόφηση από μήτρα μέσω laser σε συνδυασμό με αναλυτή μαζών χρόνου πτήσης (MALDI-TOF MS). Όλα τα ζώα στον πειραματισμό εκδήλωσαν μαστίτιδα, η οποία επιβεβαιώθηκε με βάση την απομόνωση του ενοφθαλμισμένου στελέχους και την αύξηση των σωματικών κυττάρων (κυρίως ουδετερόφιλα λευκοκύτταρα) στο γάλα, καθώς και με τα

ιστοπαθολογικά ευρήματα της παρουσίας λευκοκυτταρικής (ουδετερόφιλα λευκοκύτταρα) διήθησης, με καταστροφή του μαστικού επιθηλίου και διακυψελιδική αιμορραγία. Σε πρωτεωμικό χάρτη αναφοράς που παράχθηκε από δείγμα αίματος, που είχε συλλεχθεί πριν από τον ενοφθαλμισμό, ταυτοποιήθηκαν 19 πρωτεΐνες (σε 155 κηλίδες). Εκτός από την αλβουμίνη ορού, οι κυριότερες ταυτοποιηθείσες πρωτεΐνες ήταν η απολιποτρωτεΐνη A-I, η β-αλυσίδα ινοδωγόνου και η απτογλοβίνη - οι περισσότερες (13/20) ήταν εκκρινόμενες πρωτεΐνες, σχετίζονταν δε με φυσιολογικές λειτουργίες στα ζώα (π.χ., μεταφορά οξυγόνου). Σε πρωτεωμικούς χάρτες αναφοράς που παράχθηκαν από δύο δείγματα γάλακτος, που είχαν συλλεχθεί πριν από τον ενοφθαλμισμό, ταυτοποιήθηκαν συνολικά 40 πρωτεΐνες (σε συνολικά 280 κηλίδες). Εκτός από την αλβουμίνη ορού, οι κυριότερες ταυτοποιηθείσες πρωτεΐνες ήταν η α-λακταλβουμίνη, η α-S2-καζεΐνη, η β-λακτογλοβουλίνη και η λακτοτρανφερίνη - οι περισσότερες (17/39) ήταν εκκρινόμενες πρωτεΐνες, σχετίζονταν δε με φυσιολογικές λειτουργίες στα ζώα. Μετά τον ενοφθαλμισμό, στα δείγματα αίματος, ταυτοποιήθηκαν 33 διαφορεικά εκφρασμένες πρωτεΐνες, από τις οποίες 6 υποεκφράστηκαν, 13 εκφράστηκαν εξ αρχής (π.χ., αντιθρομβίνη-III, συμπλήρωμα C3, παράγοντας συμπληρώματος B) και 14 παρουσίασαν διακυμάνσεις στην έκφραση (π.χ., άλφα-1-αντιπρωτεΐνωση, απολιποπρωτεΐνη A-1, απολιποπρωτεΐνη A-IV, σεροτρανσφερίνη). Σε πρωτεωμικό χάρτη αναφοράς που παράχθηκε από δείγμα γάλακτος, που είχε συλλεχθεί από την ενοφθαλμισμένη μεριά του μαστού 12 ώρες μετά τον ενοφθαλμισμό, ταυτοποιήθηκαν 65 πρωτεΐνες (σε 215 κηλίδες). Εκτός από την αλβουμίνη ορού, οι κυριότερες ταυτοποιηθείσες πρωτεΐνες ήταν η κυτταροπλασματική ακτίνη-1, η β-λακτογλοβουλίνη-1/B και η καθελιδίνη-1 - οι περισσότερες (19/65) ήταν εκκρινόμενες πρωτεΐνες ή πρωτεΐνες του κυτταροσκελετού και λιγότερες (18/65) ήταν πρωτεΐνες του κυτταροπλάσματος, οι οποίες, σχετίζονταν με φυσιολογικές λειτουργίες σε ζώα ή με την αμυντική ανταπόκριση μετά από μόλυνση. Μετά τον ενοφθαλμισμό, στα δείγματα γάλακτος, ταυτοποιήθηκαν 89 διαφορεικά εκφρασμένες πρωτεΐνες, από τις οποίες 18 υποεκφράστηκαν, 53 εκφράστηκαν εξ αρχής - 3 υπερεκφράστηκαν (π.χ., άλφα-ενολάση, απολιποπρωτεΐνη A-1, καθελιδίνη-1, απτογλοβίνη, heat-shock proteins, πρωτεΐνη S100-A9, rhakinin) και 15 παρουσίασαν διακυμάνσεις στην έκφραση (π.χ., λακτοτρανσφερίνη, σεροτρανσφερίνη, τρανσθυρετίνη, tuftelin-interacting protein 11). Σε 79 πρωτεΐνες, ταυτοποιήθηκαν διαφορές στη διαφορική έκφρασή τους μεταξύ ενοφθαλμισμένης και μη ενοφθαλμισμένης πλευράς του μαστού (74 σε δείγματα από την ενοφθαλμισμένη πλευρά και 5 σε δείγματα από τη μη ενοφθαλμισμένη πλευρά του μαστού), ενώ σε 15 πρωτεΐνες ταυτοποιήθηκαν διαφορές στη διαφορική έκφρασή τους και στις δύο πλευρές του μαστού. Επίσης, 15 πρωτεΐνες (π.χ., απτογλοβίνη) εμφάνισαν διαφορική έκφραση ταυτόχρονα σε αίμα και γάλα μετά τον ενοφθαλμισμό. Σχετικά με την βιολογική διεργασία στην οποία συμμετείχαν οι πρωτεΐνες που παρουσίασαν διαφορική έκφραση, στο μεν αίμα οι

περισσότερες πρωτεΐνες συμμετείχαν στη μεταφορά ιόντων και μορίων (n=8) ή στην αμυντική απάντηση (n=7), στο δε γάλα οι περισσότερες πρωτεΐνες συμμετείχαν στην κυτταρική οργάνωση (n=17) ή στην αμυντική απάντηση (n=13).

Στο Κεφάλαιο III, μετά από σύντομη ανασκόπηση για τις καθελιδίνες (τμήμα Α), παρουσιάζεται λεπτομερής αξιολόγηση των ευρημάτων που σχετίζονται ειδικά με την καθελιδίνη-1 από τον πειραματισμό για την πρωτεωμική ανάλυση του αίματος και του γάλακτος προβατίνων σε περιπτώσεις μαστίτιδας από *M. haemolytica*, που έχει περιγραφεί στο Κεφάλαιο II (τμήμα Β). Στη συνέχεια, περιγράφεται επιπλέον πειραματισμός για τον προσδιορισμό της καθελιδίνης-1 σε γάλα προβατίνων μετά από ενδομαστικό ενοφθαλμισμό και πρόκληση μαστίτιδας (τμήμα Γ). Τέλος, υπολογίζονται συσχετίσεις μεταξύ των αποτελεσμάτων των κυτταρολογικών εξετάσεων και της ταυτοποίησης της καθελιδίνης-1 στο γάλα των προβατίνων (τμήμα Δ).

Στο Τμήμα Β, έγινε λεπτομερής αξιολόγηση των στοιχείων σχετικά με την ταυτοποίηση της καθελιδίνης-1 στα δείγματα γάλακτος στον πειραματισμό που παρουσιάζεται στο Κεφάλαιο II. Στο πλαίσιο αυτό, υπολογίστηκαν οι μέσες τιμές της οπτικής πυκνότητας των κηλίδων της καθελιδίνης-1 στα πηκτώματα, για κάθε ζώο και κάθε χρονικό σημείο δειγματοληψίας. Κηλίδες αντιπροσωπευτικές της καθελιδίνης-1 αντιστοιχήθηκαν στα πηκτώματα από τα δείγματα από κάθε ζώο για όλα τα συλλεχθέντα δείγματα. Στη συνέχεια, υπολογίστηκαν οι μέσες τιμές οπτικής πυκνότητας σε κάθε πηκτώμα. Δεν εντοπίστηκε καθελιδίνη-1 στο γάλα πριν από τον ενοφθαλμισμό. Μετά τον ενοφθαλμισμό, καθελιδίνη-1 ταυτοποιήθηκε σε δείγματα γάλακτος από την ενοφθαλμισμένη πλευρά του μαστού από τις 5/5 προβατίνες (συνολικά σε 19/22 δείγματα) για πρώτη φορά 12 ώρες μετά τον ενοφθαλμισμό, καθώς και σε δείγματα από τη μη ενοφθαλμισμένη πλευρά από 3/5 προβατίνες (συνολικά σε 5/22 δείγματα) για πρώτη φορά την 1η ημέρα μετά τον ενοφθαλμισμό. Στα δείγματα γάλακτος από την ενοφθαλμισμένη πλευρά του μαστού, η οπτική πυκνότητα των κηλίδων της καθελιδίνης-1 αυξήθηκε δραματικά ήδη στην πρώτη δειγματοληψία μετά τη μόλυνση (D0+12 ώρες) και στη συνέχεια μειώθηκε προοδευτικά ( $P=0,001$  σε σχέση με την τιμή πριν από τον ενοφθαλμισμό), επιπλέον δε οι διαφορές στα ευρήματα από την ενοφθαλμισμένη και τη μη ενοφθαλμισμένη πλευρά του μαστού ήταν σημαντικές ( $P=0,05$ ).

Στο Τμήμα Γ, παρουσιάζεται ένας πειραματισμός, στον οποίο πραγματοποιήθηκε ενδομαστικός ενοφθαλμισμός *M. haemolytica* ή *Staphylococcus chromogenes* σε προβατίνες (n=3), ο δε ετερόπλευρος μαστικός αδένας χρησιμοποιήθηκε ως μάρτυρας. Για επιβεβαίωση της ανάπτυξης μαστίτιδας και για παρακολούθηση της εξέλιξης της νόσου, χρησιμοποιήθηκαν κλινικές, μικροβιολογικές και κυτταρολογικές μέθοδοι. Δείγματα γάλακτος (για παραγωγή ορού γάλακτος) συλλέχθηκαν διαδοχικά πριν από και μετά τον ενοφθαλμισμό, συνολικά σε 5 χρονικά σημεία ανά προβατίνα μέχρι 24 ώρες μετά τον ενοφθαλμισμό. Για την πρωτεωμική ανάλυση, οι πρωτεΐνες

διαχωρίστηκαν με δισδιάστατη ηλεκτροφόρηση πηκτώματος πολυακρυλαμιδίου σε όλα τα δείγματα. Στα πηκτώματα, ανιχνεύτηκε και ταυτοποιήθηκε η καθελιδίνη-1. Επιλέχθηκαν στα πηκτώματα οι αντιπροσωπευτικές της καθελιδίνης-1 κηλίδες και η πρωτεΐνη ταυτοποιήθηκε με φασματομετρία μάζας, χρησιμοποιώντας τον φασματογράφο MALDI-TOF MS. Όλα τα ζώα στον πειραματισμό εκδήλωσαν μαστίτιδα, η οποία επιβεβαιώθηκε με βάση την απομόνωση του ενοφθαλισμένου στελέχους και την αύξηση των σωματικών κυττάρων (κυρίως ουδετερόφιλα λευκοκύτταρα) στο γάλα. Δεν ταυτοποιήθηκε καθελιδίνη-1 στο γάλα πριν από τον ενοφθαλισμό των προβατίνων. Μετά τον ενοφθαλισμό, η πρωτεΐνη ταυτοποιήθηκε στο γάλα και των τριών προβατίνων (συνολικά σε 14/15 δείγματα) από τον ενοφθαλισμένο μαστικό αδένα για πρώτη φορά 3 ώρες μετά τον ενοφθαλισμό, αλλά δεν ταυτοποιήθηκε σε κανένα δείγμα από τον ετερόπλευρο μαστικό αδένα. Η μέση τιμή της οπτικής πυκνότητας των κηλίδων της πρωτεΐνης αυξήθηκε, ήδη στο πρώτο μετά τον ενοφθαλισμό δείγμα (3 ώρες μετά τον ενοφθαλισμό), και έφτασε στη μέγιστη τιμή 12 ώρες μετά τον ενοφθαλισμό ( $P=0,002$  σε σχέση με τις τιμές πριν από τον ενοφθαλισμό), επιπλέον δε οι διαφορές στα ευρήματα από την ενοφθαλισμένη και τη μη ενοφθαλισμένη πλευρά του μαστού ήταν σημαντικές ( $P=0,05$ ).

Στο Τμήμα Δ, πραγματοποιήθηκαν υπολογισμοί και ανάλυση για διερεύνηση ενδεχόμενης συσχέτισης των τιμών της δοκιμής California Mastitis Test (CMT) ή του αριθμού των σωματικών κυττάρων με τις τιμές οπτικής πυκνότητας της καθελιδίνης-1. Για την ανάλυση, οι διάφοροι υπολογισμοί έγιναν θεωρώντας κάθε δείγμα από κάποιο ζώο ως μία παρατήρηση και έλαβε χώρα συσχέτιση αριθμητικών τιμών. Πραγματοποιήθηκε επιπλέον ανάλυση, στην οποία τα αποτελέσματα θεωρήθηκαν ως 'αρνητικά' ή 'θετικά' και έγινε η κατάλληλη συσχέτιση. Τα αποτελέσματα των δύο πειραματισμών συγκεντρώθηκαν και αναλύθηκαν συνολικά. Παρατηρήθηκε σημαντική συσχέτιση μεταξύ του κυτταρικού περιεχομένου και της οπτικής πυκνότητας των κηλίδων καθελιδίνης-1 στα δείγματα γάλακτος ( $P<0.001$ ). Η ευαυσθησία / ειδικότητα της χρήσης καθελιδίνης-1 για τη διάγνωση της μαστίτιδας ήταν 0,965 / 0,815, αντίστοιχα, ο δε θετικός προγνωστικός δείκτης / αρνητικός προγνωστικός δείκτης αυτής ήταν 0,685 / 0,980, αντίστοιχα.

Τα συμπεράσματα που προκύπτουν από τα ευρήματα αυτής της διατριβής, είναι τα παρακάτω.

(α) Μελετήθηκε το πρωτέωμα του αίματος και του γάλακτος σε προβατίνες με μαστίτιδα από *Mannheimia haemolytica*, για ταυτοποίηση και αξιολόγηση των μεταβολών στην έκφραση των πρωτεϊνών, των αλληλεπιδράσεων μεταξύ τους και των διαφοροποιήσεών τους ως αποτέλεσμα της μαστίτιδας. Τα αποτελέσματα επιβεβαίωσαν την πολυπλοκότητα μεταξύ μεσολαβητών, λευκοκυττάρων, μαστικών επιθηλιακών κυττάρων και των εκκρίσεων του μαστικού αδένος κατά την οξεία φάση της λοίμωξης. Το σύνολο των πρωτεωμικών ευρημάτων έδειξε ότι η ανοσολογική

ανταπόκριση στις προσβεβλημένες προβατίνες εξαρτάτο από πολλές πρωτεΐνες και εκδηλωνόταν μέσω διαφόρων μονοπατιών, παρατηρήθηκαν δε πολλές αλληλεπιδράσεις μεταξύ τους. Τα αποτελέσματα έδειξαν ότι ήδη 12 ώρες μετά την εναπόθεση βακτηρίων στο θηλαίο πόρο, υπήρξε εξαρχής έκφραση ή υπερέκφραση πολλών πρωτεϊνών, κυρίως στο γάλα και σε μικρότερο βαθμό στο αίμα. Από τις πρωτεΐνες στις οποίες παρατηρήθηκε εξαρχής έκφραση ή/και υπερέκφραση, ορισμένες προέρχονταν από το αίμα, ορισμένες απελευθερώθηκαν από συστατικά του αίματος που εισήλθαν στο μαστικό αδέν (π.χ., από ουδετερόφιλα λευκοκύτταρα) και ορισμένες συνετέθησαν τοπικά στο μαστικό ιστό. Θεωρήθηκε ότι οι προβατίνες προσπαθούσαν να διατηρούσαν την σύνθεση και παραγωγή γάλακτος, ταυτοχρόνως δε να εξυπηρετούσαν τις ανάγκες για ανοσολογική ανταπόκριση και λευκοκυτταρική δραστηριότητα. Υπήρχαν ενδείξεις ότι αρκετές πρωτεΐνες θα μπορούσαν να χρησιμοποιούνταν ως βιοδείκτες για τη μαστίτιδα στις προβατίνες.

(β) Η ανίχνευση καθελιδίνης-1 στο γάλα συσχετίστηκε με αυξημένη ευαισθησία και ειδικότητα στη διάγνωση της μαστίτιδας. Υπήρχε αυξημένη συσχέτιση της παρουσίας της καθελιδίνης-1 με τα αποτελέσματα της κυτταρολογικής εξέτασης, τα αποτελέσματα δε ήταν παρόμοια όταν τα ευρήματά της ελήφθησαν υπόψη ως ποσοτικά (αριθμητικές τιμές) ή ποιοτικά ('θετικά'/'αρνητικά') αποτελέσματα. Η καθελιδίνη-1 ανιχνεύτηκε στο γάλα πιο νωρίς από την αύξηση του αριθμού των σωματικών κυττάρων. Η ανίχνευση της καθελιδίνης-1 για την διάγνωση της μαστίτιδας έχει το πλεονέκτημα ότι, καθώς δεν ανιχνεύεται στο γάλα των υγιών προβατίνων, δεν υπάρχει ανάγκη να καθιερωθεί ουδός για την τιμή της, αλλά θα αρκούσε μία αξιολόγηση 'θετικό'/'αρνητικό'.

**Με βάση την κείμενη νομοθεσία και μετά από σχετική απόφαση στη με αριθμό 30/27.01.2016 συνεδρίαση της Γενικής Συνέλευσης Ειδικής Σύνοψης του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας, η συγγραφή της διατριβής έγινε στην αγγλική γλώσσα.**

## Δημοσιεύσεις σχετιζόμενες με την παρούσα διατριβή

Στις παρακάτω επιστημονικές δημοσιεύσεις παρουσιάζονται τμήματα της παρούσας διατριβής:

The following scientific papers presenting facets of the present thesis, are available:

- I. A.I. Katsafadou, G.C. Fthenakis, A.K. Anagnostopoulos, C. Billinis, M.S. Barbagianni, S.A. Spanos, V.S. Mavrogianni, G.T. Tsangaris (2015) "Proteomic analysis of blood and milk of ewes with experimental mastitis induced by *Mannheimia haemolytica*" *Proceedings of the 9th Annual Congress of European Proteomics Association (Milan, Italy)*, p. 56.
- II. A.I. Katsafadou, G.T. Tsangaris, C. Billinis, G.C. Fthenakis (2015) "Use of proteomics in the study of microbial diseases of small ruminants" *Veterinary Microbiology*, 181:27-33.
- III. A.I. Katsafadou (2016) "From veterinary science to 'Veterinomics'" *SM Journal of Bioinformatics and Proteomics*, 1(1):1003.
- IV. A.I. Katsafadou, G.T. Tsangaris, C. Billinis, G.C. Fthenakis (2016) "Applied proteomics in companion animal medicine" *Current Proteomics*, 13:165-171.
- V. A.I. Katsafadou, G.T. Tsangaris, N.G.C. Vasileiou, K.S. Ioannidi, C. Billinis, G.C. Fthenakis (2017) "Identification of cathelicidin-1 in milk of ewes with mastitis and its potential significance in diagnosis of subclinical mastitis" *Proceedings of the 9th International Sheep Veterinary Congress (Harrogate, United Kingdom)*, δεκτό για παρουσίαση.



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SCHOOL OF HEALTH SCIENCES  
FACULTY OF VETERINARY MEDICINE

**PROTEOMIC STUDY OF OVINE MASTITIS  
ASSOCIATED WITH *MANNHEIMIA HAEMOLYTICA***

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**A THESIS SUBMITTED FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY**

Work carried out at the Department of Obstetrics and Reproduction  
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# ABSTRACT

Specific objectives of the present thesis were as follows: (i) the study of differentially expressed proteomes in ewes after experimentally induced *Mannheimia haemolytica* mammary infection and (ii) the study of the presence of cathelicidin-1 in milk as a diagnostic indicator for detection of subclinical mastitis in sheep, using infection models.

The thesis is divided into three chapters followed by the General Discussion.

In Chapter I, the relevant literature is reviewed. The Chapter is subdivided into two Parts. In Part A, the literature on methodologies in proteomics is briefly reviewed. In Part B, the literature in applied proteomics in veterinary science is presented.

In Chapter II, after a brief review regarding *M. haemolytica* and its role in mastitis (Part A), an experiment detailing the proteomics analysis of blood and milk of ewes with induced *M. haemolytica* mammary infection is presented (Parts B and C).

Mastitis was induced in lactating ewes (n=5) by deposition of a pathogenic *M. haemolytica* strain into the teat duct of ewes; the contralateral side of the udder was used as control. In order to confirm infection and to monitor progress of the disease, standard clinical, microbiological, cytological and histopathological methods were employed. Samples of blood (for plasma extraction) and milk (for whey extraction) were collected sequentially before and after challenge, in total, at 6 or 7 time-points per ewe up to 4th day post-challenge. For proteomics study, proteins were separated by two-dimensional gel electrophoresis (2-DE) for all samples. Differentially expressed proteins were detected semi-automatically in 2-DE gels. Spots were picked and the protein content was identified by using Matrix-assisted laser desorption/ionisation Time-of-flight mass spectrometre (MALDI-TOF MS). Animals developed mastitis, confirmed by isolation of the challenge strain and increase of cellular content (predominantly neutrophils) in milk and by histopathological evidence of leucocytic (neutrophils) infiltration with mammary epithelial destruction and intra-alveolar haemorrhages. In a protein reference map produced from a blood sample collected before inoculation, 19 proteins (in 155 spots) were identified; apart from serum albumin, apolipoprotein A-I, fibrinogen beta chain and haptoglobin were the predominant proteins on the gel; most (13/20) proteins were secreted and, in general, were involved in roles related to physiological functions in healthy animals (e.g., oxygen transport). In protein reference maps produced from two milk samples collected before inoculation, in total, 40 proteins (in totally 280 spots) were identified; apart from serum albumin, alpha-lactalbumin, alpha-S2-casein, beta-

lactoglobulin-1/B and lactotransferrin were the predominant proteins on the gels; most (17/39) proteins were secreted and, in general, were involved in roles related to physiological functions in healthy animals. After challenge, in blood samples, 33 differentially expressed proteins were identified; of these, 6 were observed with downregulation, 13 with new expression (e.g., antithrombin-III, complement C3, complement factor B, complement C3, complement factor B) and 14 with fluctuating pattern (e.g., alpha-1-antiproteinase, apolipoprotein A-I, apolipoprotein A-IV, serotransferrin). In a protein reference map produced from one milk sample from inoculated side of the udder collected 12 h after inoculation, in total, 65 proteins (in 215 spots) were identified; apart from serum albumin, actin cytoplasmic 1, beta-lactoglobulin-1/B and cathelicidin-1 were the predominant proteins on the gels; most (19/65) proteins were secreted or cytoskeleton proteins, with fewer being cytoplasm proteins (18/65); they were involved in roles related to physiological functions or associated with the post-infection defence response in animals. After challenge, in milk samples, 89 differentially expressed proteins were identified; of these 18 were observed with downregulation, 53 with new expression – 3 with upregulation (e.g., alpha-enolase, apolipoprotein A-I, cathelicidin-1, haptoglobin, heat shock proteins, haemoglobins, protein S100-A9, phakinin) and 15 with fluctuating pattern (e.g., lactotransferrin, serotransferrin, transthyretin, tuftelin-interacting protein 11). Varying differential expression in samples from inoculated and non-inoculated side of the udder were recorded in 79 proteins (74 were differentially expressed only in samples from the inoculated side and 5 were differentially expressed only in samples from the non-inoculated side), whilst in 15 proteins differential expression was observed in samples from both sides of the udder. In total, 15 proteins (e.g., haptoglobin) showed status changes in both blood and milk of inoculated side of the udder after challenge. Regarding the biological process in which differentially expressed proteins were involved, in blood most proteins were involved in transport of ions and molecules (n=8) or in inflammatory and defence response (n=7); in milk from inoculated side, most were involved in cell organisation and biogenesis (n=17) or in inflammatory and defence response (n=13).

In Chapter III, after a brief review regarding cathelicidins (Part A), detailed evaluation of findings related specifically to cathelicidin-1 obtained in the experiment described in Chapter II is presented (Part B). Then, the identification of cathelicidin-1 in milk of ewes with induced mammary infection is described (Part C). Finally, associations between results of cytological examinations and identification of cathelicidin-1 in milk of ewes are calculated (Part D).

In Part B, detailed data regarding cathelicidin-1 identification, obtained during processing of milk samples in the experiment described in Chapter II is presented. Mean optical densities of cathelicidin-1 spots on 2-DE gels, obtained for each ewe, on each sampling point were calculated.

Spots representing cathelicidin-1 were matched across gels obtained from samples collected from the same ewe throughout the study. Subsequently, means were calculated for results obtained for each animal on each sampling point. Presence of cathelicidin-1 in milk was not evident before inoculation of ewes. After challenge, the protein was recorded in samples from 5/5 ewes (19/22 samples) from the inoculated side of the udder, starting 12 h (D0+12 h) after inoculation, as well as in 3/5 ewes (5/22 samples) from the contralateral side of the udder, starting on the 1st day after inoculation. In milk samples from the inoculated side of the udder of all ewes, spot density of cathelicidin-1 increased sharply, starting at the first post-inoculation sampling (D0+12 h) and progressively decreased thereafter ( $P=0.001$ ), with differences between results of inoculated and non-inoculated glands being significant ( $P=0.05$ ).

In Part C, mastitis was induced by intramammary inoculation of *M. haemolytica* or *Staphylococcus chromogenes* in ewes ( $n=3$ ); the contralateral side of the udder was used as control. In order to confirm infection and to monitor progress of the disease, standard clinical, microbiological and cytological methods were employed. Samples of milk (for whey extraction) were collected sequentially before and after challenge and until 24 h after that. For proteomics study, proteins were separated by 2-DE for all samples. Cathelicidin-1 was identified semi-automatically in 2-DE gels. Spots were picked and the protein content was identified by using MALDI-TOF MS. Animals developed mastitis, confirmed by isolation of the challenge strain and increase of cellular content (predominantly neutrophils) in milk samples. Presence of cathelicidin in milk was not evident before inoculation of ewes. After challenge, it was recorded in samples from 3/3 ewes (14/15 samples) from the inoculated side of the udder, starting 3 h after inoculation, but from no sample from the contralateral side of the udder. In milk samples from the inoculated side of the udder of all ewes, spot density of cathelicidin-1 increased sharply, starting at the first post-inoculation sampling (3 h after inoculation) and progressively increased further until 12 h after inoculation ( $P=0.002$ ), with differences between results of inoculated and non-inoculated glands being significant ( $P=0.05$ ).

In part D, computations are performed to evaluate correlation between California Mastitis Test (CMT) scores or somatic cell counts and optical densities of cathelicidin-1 in milk samples. For calculations, results obtained from each ewe on the respective occasion were considered. Analysis of correlation, with results considered as 'negative' or 'positive', was also performed. Results of both experiments were considered together. There was significant correlation between cellular content and cathelicidin-1 spot densities in milk samples ( $P<0.001$ ). Sensitivity / Specificity of using detection of cathelicidin-1 for diagnosis of mastitis was 0.965 / 0.815, respectively, with positive predictive value / negative predictive value being 0.685 / 0.980.

The conclusions from the results of the present thesis are summarised herebelow.

(a) The proteome of blood and milk of ewes with mastitis associated with *Mannheimia haemolytica* has been studied, for identification and evaluation of changes in protein expression, interactions or modifications as the result of mastitis. The findings confirmed the complex interactions between mediators, leucocytes, mammary cells and lacteal secretions that took place during acute inflammatory reaction in mastitis. The entirety of proteomics findings has indicated that affected ewes had mounted a defence response that had been regulated by many proteins and through various pathways; these were interdependent at various points. The results have indicated that already 12 hours subsequently to bacterial deposition into the teat duct, new expression and/or upregulation of increased number of proteins was evident, primarily in milk and to a lesser degree in blood. Of the proteins observed with new expression or upregulation in milk, some were of blood origin, some were released by blood constituents that entered into the mammary gland (e.g., by neutrophils) and some were locally synthesized in the mammary gland. It may be postulated that ewes attempted to continue milk synthesis and production, as well as accommodating the increased needs for leucocytic activities. There were indications that various proteins might be of value as biomarkers for ovine mastitis.

(b) Detection of cathelicidin-1 in milk has been associated with increased sensitivity and specificity in diagnosis of mastitis. There was an increased correlation of presence of cathelicidin-1 with results of cytological examination; similar findings were recorded when cytological results were taken into account as either quantitative (numerical values) or qualitative (positive/negative) results. Cathelicidin-1 was detected in milk earlier than increased cellular content. When used for diagnosis of mastitis, cathelicidin-1 has the advantage that, as it is not present in milk of healthy ewes, there would be no need to establish a threshold, hence a 'positive'/'negative' assessment would suffice.

## **Publications associated with the present thesis**

The following scientific papers presenting facets of the present thesis, are available:

I. A.I. Katsafadou, G.C. Fthenakis, A.K. Anagnostopoulos, C. Billinis, M.S. Barbagianni, S.A. Spanos, V.S. Mavrogianni, G.T. Tsangaris (2015) "Proteomic analysis of blood and milk of ewes with experimental mastitis induced by *Mannheimia haemolytica*" *Proceedings of the 9th Annual Congress of European Proteomics Association (Milan, Italy)*, p. 56.

- II.** A.I. Katsafadou, G.T. Tsangaris, C. Billinis, G.C. Fthenakis (2015) "Use of proteomics in the study of microbial diseases of small ruminants" *Veterinary Microbiology*, 181:27-33.
- III.** A.I. Katsafadou (2016) "From veterinary science to 'Veterinomics'" *SM Journal of Bioinformatics and Proteomics*, 1(1):1003.
- IV.** A.I. Katsafadou, G.T. Tsangaris, C. Billinis, G.C. Fthenakis (2016) "Applied proteomics in companion animal medicine" *Current Proteomics*, 13:165-171.
- V.** A.I. Katsafadou, G.T. Tsangaris, N.G.C. Vasileiou, K.S. Ioannidi, C. Billinis, G.C. Fthenakis (2017) "Identification of cathelicidin-1 in milk of ewes with mastitis and its potential significance in diagnosis of subclinical mastitis" *Proceedings of the 9<sup>th</sup> International Sheep Veterinary Congress (Harrogate, United Kingdom)*, accepted for presentation.

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# TABLE OF CONTENTS

	Page
<b>ΠΕΡΙΛΗΨΗ</b>	3
<b>ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ - ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ</b>	9
<b>ABSTRACT</b>	11
<b>ADVISORY COMMITTEE - EXAMINATION BOARD</b>	16
<b>TABLE OF CONTENTS</b>	17
<b>GENERAL INTRODUCTION</b>	20
Preface - Objectives of the thesis	21
Acknowledgments - Ευχαριστίες	27
<b>CHAPTER I</b>	30
<b>REVIEW OF THE LITERATURE</b>	
<b>A. METHODOLOGIES IN PROTEOMICS - GENERAL APPRAISAL</b>	31
Protein separation techniques	31
Mass spectrometry (MS)	36
Bioinformatics	41
<b>B. APPLIED PROTEOMICS IN VETERINARY MEDICINE</b>	45
Applied proteomics in small ruminant health management	45
Applied proteomics in health management of other farm animal species	54
Applied proteomics in canine and feline medicine	60
Applied proteomics in medicine of equids	66
Applied proteomics in health management of rabbits	68

<b>CHAPTER II</b>	<b>70</b>
<b>PROTEOMICS ANALYSIS OF BLOOD AND MILK OF EWES</b>	
<b>WITH INDUCED <i>MANNHEIMIA HAEMOLYTICA</i> MAMMARY INFECTION</b>	
<b>A. INTRODUCTION</b>	<b>71</b>
<b>B. MATERIALS AND METHODS</b>	<b>74</b>
Experimental overview	74
Inoculation procedure	76
Pre- and post-inoculation procedures in the experimental ewes	76
Conventional laboratory examinations in samples	
from the experimental animals	78
Preliminary processing of samples for proteomics examination	79
Measurement of protein content of samples by Bradford protein assay	80
First dimension separation: isoelectric focusing (IEF)	80
Second dimension separation: SDS-PAGE	84
Protein identification	88
Data management and analysis	93
Appendix: Details of preparations used in the study	98
<b>C. RESULTS</b>	<b>101</b>
Results of clinical and conventional laboratory examinations	
in samples from the experimental animals	101
Results of proteomics examinations in samples	
from the experimental animals	107
<b>CHAPTER III</b>	<b>158</b>
<b>SIGNIFICANCE OF CATHELICIDIN-1 IN MILK OF EWES</b>	
<b>WITH INDUCED <i>MANNHEIMIA HAEMOLYTICA</i> MAMMARY INFECTION</b>	
<b>A. INTRODUCTION</b>	<b>159</b>
<b>B. DETAILED EVALUATION OF FINDINGS RELATED TO CATHELICIDIN-1</b>	
<b>IN EXPERIMENT OF ‘PROTEOMICS ANALYSIS OF BLOOD AND MILK</b>	
<b>OF EWES WITH INDUCED <i>MANNHEIMIA HAEMOLYTICA</i></b>	
<b>MAMMARY INFECTION’</b>	<b>162</b>
Materials and methods	162
Results	163

<b>C. IDENTIFICATION OF CATHELICIDIN-1 IN MILK OF EWES WITH INDUCED MAMMARY INFECTION IN A BLINDED EXPERIMENT</b>	168
Materials and methods	168
Results	172
<b>D. ASSOCIATIONS BETWEEN RESULTS OF CYTOLOGICAL EXAMINATIONS AND IDENTIFICATION OF CATHELICIDIN-1 IN MILK OF EWES</b>	180
Materials and methods	180
Results	181
<b>GENERAL DISCUSSION</b>	187
Introduction	188
Proteomics findings after deposition of <i>Mannheimia haemolytica</i> into the teat duct of ewes	189
Potential use of cathelicidin-1 in the diagnosis of <i>Mannheimia haemolytica</i> -associated mastitis	208
Epilogue	212
<b>REFERENCES</b>	214

# **GENERAL INTRODUCTION**

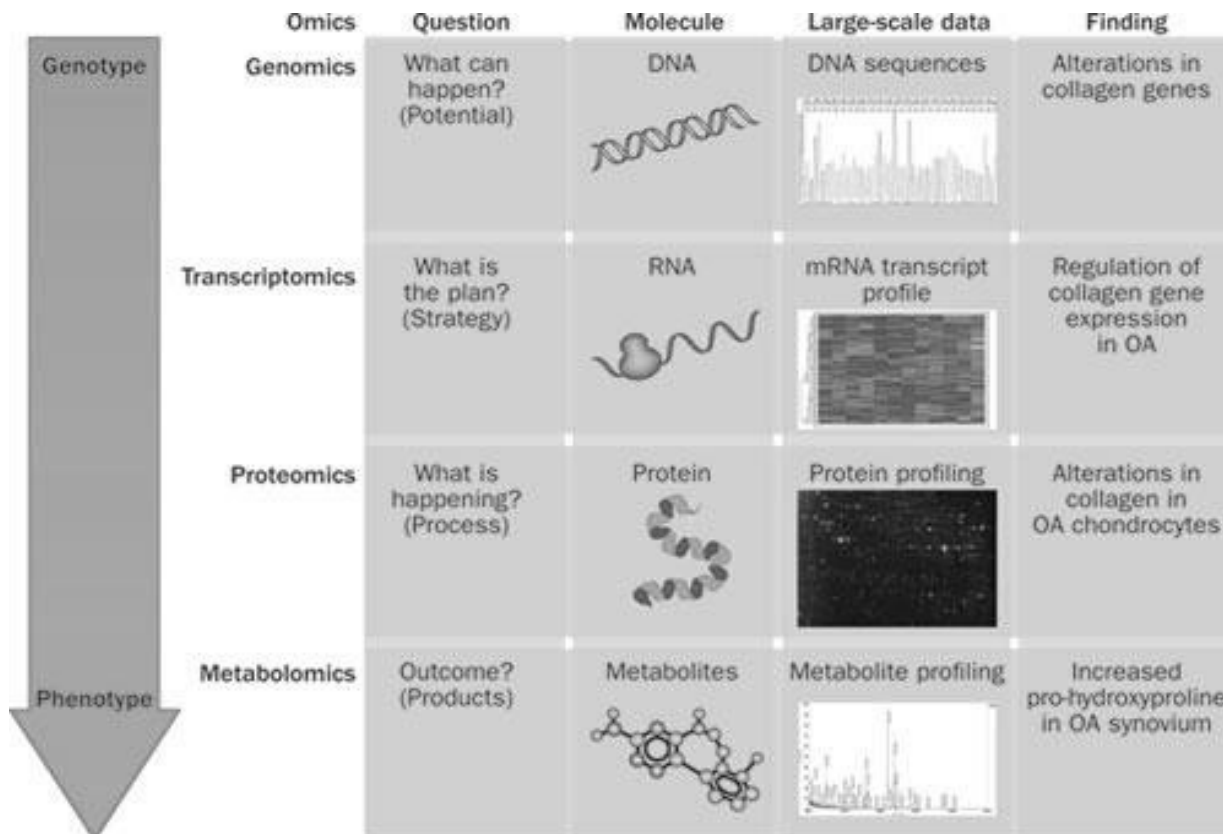
## Preface - Objectives of the thesis

# Introduction to proteomics

## Preamble

Genome is the collection of all genes in an organism. In a related approach, transcriptome can be defined as the collection of all genes expressed at mRNA level and their translated proteins. Further, the proteome is the entire set of proteins and describes all proteins that are encoded by the genome (PROTEin+genOME) in a cell or a tissue at any given time, which takes into account all post-translational modifications (Wilkins et al. 1996) (Figure G1.1.) An organism's genome is stable and well defined; the transcriptome and the proteome are highly dynamic and change depending on various physiological states and pathological conditions occurring in a particular tissue of that organism. Proteomics is the step subsequent to genomics and transcriptomics in the study of biological systems.

**Figure G1.1.** The ‘omics’ cascade: important branches of ‘omics’ with their major components being used at various molecular levels to reveal gene expression (modified from Fisher 2005).



The objective of proteomics work is the identification of proteins of interest present in a sample and the quantification of changes in their expression as the result of varying physiological states or of different pathological conditions. There are several technological approaches to achieve this aim, which depend upon the type of sample and the equipment available.

The genome of mammals consists of about 30,000 genes (Claverie 2001). Hence, mammals produce a considerably complex array of proteins from their genome. The high complexity in mammalian proteomes is the consequence of various factors, which include alternative splicing of mRNA, multiple pathways of proteolytic processing of proteins, as well as various other post-translational modifications (e.g., glycosylation, prenylation, phosphorylation). Moreover, the subcellular localisation of a protein may affect its function (Petsko 2001). These mechanisms exist in all eukaryotic organisms, but are stronger in some taxonomic classes, such as the mammals, that way producing more complex transcriptomes and proteomes.

#### *'Omics' technologies*

Technologies that measure some characteristic of a large family of cellular molecules (e.g., genes, proteins, metabolites) have been collectively described by using the suffix '-omics' at the end of the name of the characteristic measured. Etymology of the suffix comes from the Latin suffix '-ome', meaning mass or many. Currently, the terminology '-omics' refers to technologies, which explore roles of various molecules that make up the cells of an organism, their actions, as well as the relationships between them (Lerias et al. 2014). Cumulatively, all these technologies are termed '*biomics*', as they enable study of organisms at different levels (Athanasiadou and Huntley 2008). Biomics include the following specific technologies: (i) genomics, i.e. the study of genome(s) which includes the study of genes and their function, (ii) transcriptomics, i.e. the study of transcriptome(s) focusing on the analysis of mRNA, (iii) proteomics, i.e., the study of all proteins (proteome) and their interactions in a cell or tissue at a given time, (iv) glycomics, i.e., the study of cellular carbohydrates, (v) lipidomics, i.e. the study of cellular lipids, and (vi) metabolomics, i.e. the study of metabolomes which represent the collection of all metabolites in a biological organism as end products of gene expression and cellular metabolism.

Genomics projects have greatly benefited by using techniques such as polymerase chain reaction (PCR) or microarray and have provided information about gene sequence. Gene sequence, transcription, translation and post-translation are all significant in the expression of genes, for the study of which the following two approaches can be used: (i) quantification of mRNA and (ii) detection or relative quantification of protein. Measurement of mRNA and/or protein can be

accomplished by means of techniques measuring single genes (e.g., real-time PCR for mRNA, Western blot for proteins) or genome-wide technologies measuring thousands of genes (e.g., microarray for mRNA, proteomics for proteins). Microarray analysis is used to determine mRNA expression for as many as 10,000 to 40,000 different genes (Stoughton 2005, Ness 2007). The method has the advantage of being able to measure gene expression changes in a large proportion of genes of one cell in only one experiment. However, lack of correlation between mRNA and protein abundance has now been repeatedly confirmed (Gygi et al. 1999, Ideker et al. 2001, Griffin et al. 2002).

Proteomics refers to the large-scale study of protein expression, protein-protein interactions or post-translational modifications (Gingras et al. 2007, Witze et al. 2007). Unlike other methodologies (e.g., Western blot) which can analyse only a few proteins at a time, by using proteomics techniques and methodologies one may study hundreds of proteins in a single experiment. The technique can show the dynamics of cellular response to changes in their micro-environment within tissues. Therefore, it is possible to identify changes in protein expression, interaction or modification, which occur as a result of changes in physiological states and of pathological conditions occurring at that tissue within that organism. Generation of large proteomics data sets may be used to demonstrate interdependence of the various cellular processes, which are of importance in the normal cell growth or the cellular response to abnormal situations or disease conditions. As a result, by using a proteomics approach, an investigator can view as one picture, in its entirety, the cellular action and response, rather than examining the individual role of each protein separately. Such an experimental approach enables discovery of associations between cellular processes, which may be used as precursors to new hypotheses.

#### *An overview of technologies and approaches applied in proteomics work*

A great variety of animal samples has been used for application of proteomics methodologies; these include, but are not limited to, blood (serum, plasma or blood constituents from adult or young animals or foetuses), lymph, saliva, tears, gastric mucin, rumen content, bile, bronchoalveolar lavage, cerebrospinal fluid, seminal plasma, uterine content from gravid (allantoic fluid, amniotic fluid) or non-gravid uteri and milk (whole milk, milk whey, milk fat globules, colostrum), as well as various tissues (e.g., brain, intestine, lung, adipose tissue). Moreover, various pathogens have been studied by means of proteomics techniques; wild or vaccinal strains have been included in such studies.

Various techniques and methods are available in proteomics methodology. These include gel-based proteomics (e.g., two-dimensional polyacrylamide gel electrophoresis [2-D PAGE]) and

shot-gun proteomics (e.g., high-resolution liquid chromatography [HPLC], liquid chromatography in combination with mass spectrometry [LC MS] or nano-capillary LC MS [nano LC MS]), which lead, by means of bioinformatics approaches, to protein identification (Palagi et al. 2006, Goerg et al. 2009, Mazzucchelli and De Pauw 2013). However, technical limitations may interfere with complete elucidation of the proteome, e.g. use of 2-D PAGE leads to under-representation of hydrophobic proteins in the proteome (Cash 2011).

Mass spectrometry (MS) is a technique employed in analytical chemistry, which supports the identification of the type and the amount of chemicals present in a sample. Its principle is based in measuring the mass-to-charge ratio and the abundance of gas-phase ions (Aebersold and Mann 2003).

There are two general approaches used in protein identification. In simple experiments, digestion of sample proteins by using an enzyme, e.g., trypsin, will result in fragmenting the protein(s) to peptides of varying masses. This procedure is termed 'peptide mass fingerprinting' and can be completed by means of a mass spectrometre (e.g., matrix assisted laser desorption/ionisation time-of-flight mass spectrometre [MALDI-TOF]). In more complex experimental procedures, enzyme-digested proteins are fragmented (by means of mass spectrometry) to ions, which can be used to predict the amino acid sequence of each peptide. This approach uses a process termed 'collision-induced dissociation' to fragment the peptides, which may be performed successfully only with tandem mass spectrometres. Selection of the appropriate approach on each occasion is dependent upon the type of sample under evaluation, the objective of the study and the available equipment (Panisko et al. 2002, Domon and Aebersold 2006).

Various approaches and/or techniques for protein identification and/or quantification are in use nowadays. The three major procedures are difference gel electrophoresis, isotope-coded affinity tags and isobaric tag for relative and absolute quantitation (Lambert et al. 2005, Bantscheff et al. 2007, Karp and Lilley 2007). Quantitative proteomics may be employed for the identification of a protein, as well as its expression in samples from animals in varying physiological states or health conditions.

### *Applications of proteomics work*

#### General considerations

Use of the various methods is within the frame of the various approaches employed at respective circumstances. *In vitro* works include studies of microbial cells and interactions between cells or host analyses; these are advantageous in providing regulated conditions and may be used



in identifying protein biomarkers associated with microbial virulence. However, they clarify only a limited spectrum of the microbial proteome, as many genes are expressed and various proteins are identifiable only during host invasion. This can be partly rectified by employing '*in vivo* mimicking conditions', e.g. for gastrointestinal tract pathogens creating the acid-stress response (Birch et al. 2003), which can be of substantial usefulness. In contrast, use of proteomics under *in vivo* conditions in infectious diseases leads to the elucidation of protein biomarkers in pathogenetic processes. It is noteworthy that in the latter approach, lack of fully sequenced genomes in many livestock species may prove to be a limiting factor during usage of '*omics*' technologies, hence searches of nucleotides or peptide sequences in tissue samples may fail to provide significant hits (Athanasiadou and Huntley 2008, Deutsch et al. 2008); moreover, lack of an amplification technique for proteins, which abound at tissue level during host invasion, is another limiting factor in those studies.

#### Specific approaches

Proteomics may be used to survey proteins expressed in cellular compartments, in cells, in tissues (including biological fluids), which can yield data libraries regarding the abundant proteins (Lippolis and Reinhardt 2005, Radosevich et al. 2007). Some of the new proteins found during the process will lead to future hypothesis-driven research. Further, elucidation of normal processes can be supported by application of proteomics methodologies. Quantitative proteomics may be employed for the identification of a protein, as well as its expression in samples from animals in varying physiological states or health conditions. Moreover, results of descriptive studies in healthy animals can be used for reference, as they provide baseline measurements and reference values in healthy animals.

Significant generalistic examples of applications of the technologies include the identification of difference between healthy and diseased animals and the identification of properties of bacteria involved at a disease process compared to those of the same organisms at *in vitro* conditions. Proteins and/or pathways that have been found to change during the above situations (Boyce et al. 2006, Lippolis et al. 2006), can be targets for future research. This approach can be helpful in studying host-pathogen interactions for the identification of animal or bacterial proteins of significance in disease process and recovery, in organ development or in host response to stress, which can be performed by examination of sample material from diseased hosts (Eckersall et al. 2012). Proteomics can also facilitate improved diagnosis of diseases by identifying biomarkers indicative of subclinical problems (Lamy and Mau 2012, Gutiérrez et al. 2013). Usually, a large array of proteins can be identified as biomarkers of respective phenotypic

virulence characteristics. These should be later correlated to specific pathogenic effects in the host(s).

Further, proteomics technologies have been used widely in the study of expression of genes and physiological processes by microbes (Cash 2011), for example in identification of protein biomarkers and/or in studying host-pathogen interactions. Proteins which are significantly modified in their expression and/or location or proteins modified post-translationally in animals with a pathological condition compared to healthy animals, can be studied for inclusion in diagnostic tests or in new treatment procedures.

Moreover, development of novel vaccines may also advance by use of proteomics. In that work, the functional genomics stage deals with experimental identification and selection of target proteins using proteomics techniques (Mohavedi and Hampson 2008). In a proteomics approach in vaccine development, bacterial proteins (e.g., surface proteins -‘surfaceome’; Cullen et al. 2005), are first resolved into their individual components, followed by digestion of each protein into its peptide fragments. Finally, the generated peptide fingerprint is used for input into a database search of predicted masses arising from digestion of a list of known proteins (Mohavedi and Hampson 2008).

## Objective of the thesis

Veterinary proteomics is a developing field with the aim to generate knowledge for subsequent clinical applications. The field has not been as developed as medical proteomics, primarily because of financial limitations. Nevertheless, there is a scope for growth, as proteomics use will contribute to the elucidation of mechanisms, will support control of various diseases and will improve management practices.

Mastitis is the most significant disease of the udder of ewes, causing extensive financial losses, particularly in dairy sheep flocks. Recently, mastitis has been described as the most significant welfare problem in sheep, independently of production type and management system (European Food Safety Authority 2014).

The present thesis focusses in the proteomics of ovine mastitis, by using a working model mastitis associated with *Mannheimia haemolytica*. The general objective of the thesis is to increase available knowledge regarding proteomics of ovine mastitis. Specific objectives of the thesis are as follows.

- The study of differentially expressed proteomes in ewes after experimentally induced *M. haemolytica* mammary infection.

- The study of the presence of cathelicidin in milk as a diagnostic indicator for detection of subclinical mastitis in sheep.

The present thesis has been carried out at the Department of Obstetrics and Reproduction of the Veterinary Faculty of the University of Thessaly. All the proteomics laboratory work described in the thesis have been performed at the Proteomics Unit of Biomedical Research Foundation Academy of Athens. Research work started in 2013 and was carried out until mid-2016; it was followed by analysis of results and writing up of the thesis. The thesis was financially supported by departmental funds, as well as by funds of the Proteomics Unit of Biomedical Research Foundation Academy of Athens.

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# **CHAPTER I**

## **REVIEW OF THE LITERATURE**

## **A. METHODOLOGIES IN PROTEOMICS – GENERAL APPRAISAL**

### **Protein separation techniques**

In order to enlight the proteome, fractionation techniques are used, which can be electrophoretic or chromatographic. Electrophoretic techniques are applied to intact proteins, while chromatographic ones to peptides that have been generated after previous protein cleavage by enzymatic or chemical techniques; the combination of techniques has been also applied, which increased their efficacy (Xie et al. 2011).

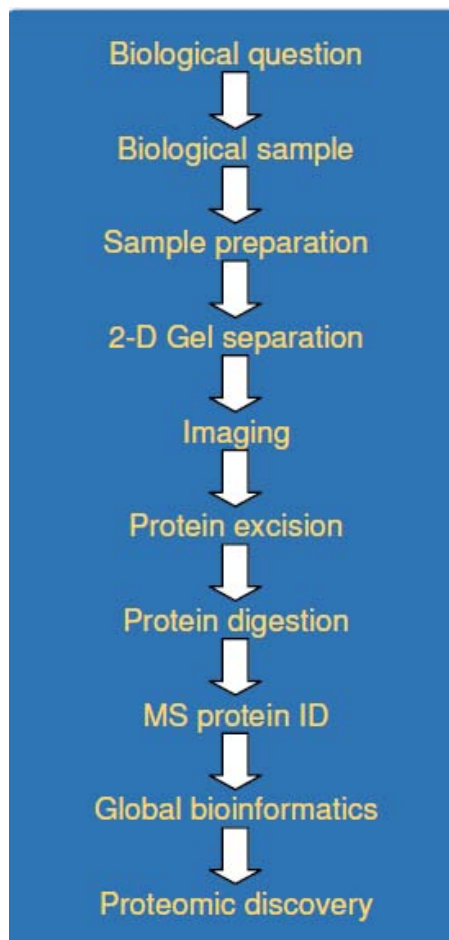
### **Gel-based proteomics**

There are several types of gel electrophoresis procedures, which are common in proteomics experiments. For example, one-dimension protein electrophoresis of blood serum samples separates blood serum proteins on agarose gels on the basis of their molecular size and electric charge (Stockham and Scott 2008); by employing blood serum protein electrophoresis, one can produce an electrophoretogram, which is a graphical representation of the protein constituents of blood serum in groups of proteins or bands; however, one-dimension blood serum protein electrophoresis does not provide a good resolution in terms of separation of blood serum proteins (Smithies and Poulik 1956).

The most common type of gel electrophoresis associated with proteomics work is the two-dimensional polyacrylamide gel electrophoresis (2-D PAGE or 2-DE) (Goerg et al. 1988, 2000). By means of this technique, it is possible to image over 1,800 proteins in a single gel (Choe and Lee 2000); therefore, it is a powerful primary tool of proteomics research, especially when requiring to separate multiple proteins for parallel analysis. That way, the technique allows up to thousands of gene products to be analysed at the same time. Moreover, it is possible, in combination with computer-assisted image evaluation systems for examination of proteomes, to list and compare data obtained at different times or locations.

The general workflow in a conventional two-dimensional polyacrylamide gel electrophoresis gel-based proteomics experiment is described in further detail herebelow and summarised in Figure I.1. During the work, various factors influence the way that the experiment is performed and various factors may affect the results.

**Figure I.1.** Flow chart of work in a two-dimensional gel-based proteomics experiment with factors that may affect the experiment and some factors that may influence the way the experiment is performed (Garfin and Heerdt 2001).



### *Sample preparation*

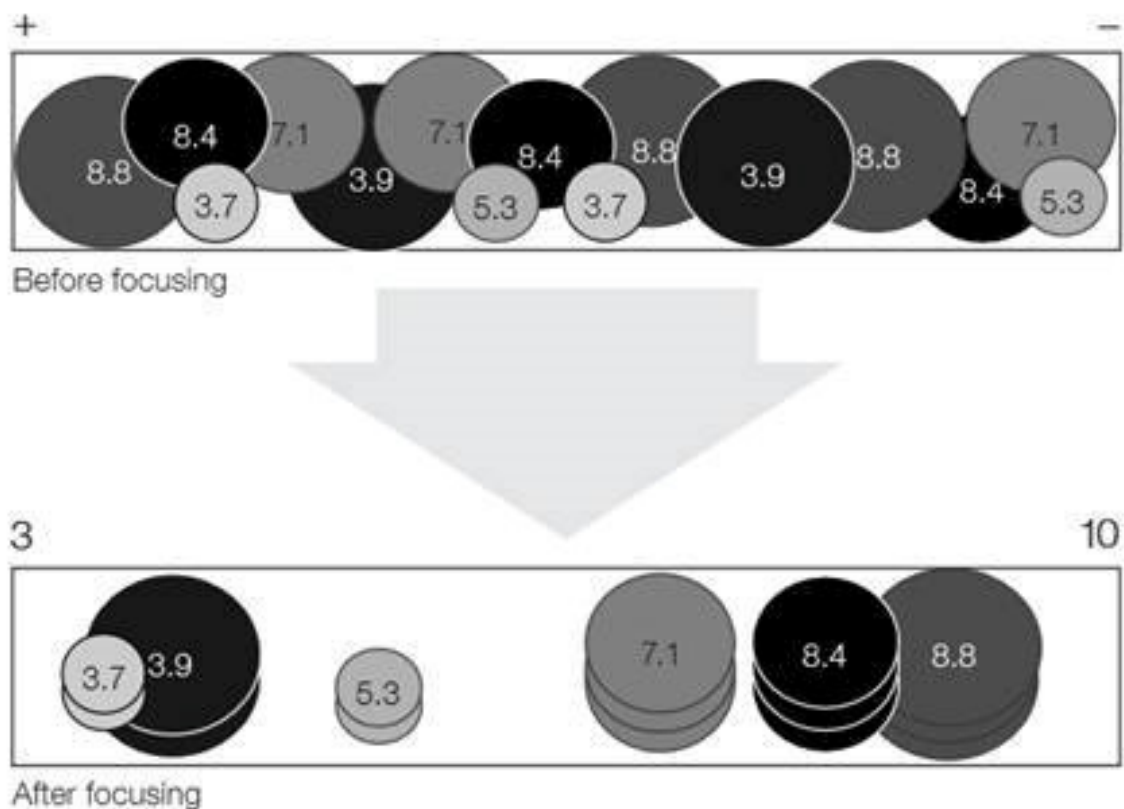
Sample preparation is important for success of the experiment and depends upon the objective of research. The whole procedure depends on the solubility, the size, the charge and the isoelectric point of the proteins in the sample under consideration. Sample preparation is also important in reducing the complexity of protein mixture. The protein fraction to be loaded on a gel for two-dimensional electrophoresis must be in a low ionic strength denaturing buffer, which is able to maintain the charge of proteins and to keep them soluble (Link 1999).



### *First-dimension separation*

During first-dimension separation, proteins are separated on the basis of their isoelectric point; this is defined as the pH at which a protein carries no net charge and will not migrate in an electrical field. In first dimension separation, the technique is termed 'isoelectric focusing' (Issaq and Veenstra 2008); for 2-D PAGE, the method is best performed in immobilised pH gradients, usually termed 'IPG strips' (Gianazza and Righetti 2009). These help to differentiate complex protein mixtures across a wide range of pH (Bjellqvist et al. 1982) (Figure I.2.).

**Figure I.2.** A mixture of proteins is resolved on a pH 3-10 IPG strip according to each protein's isoelectric point and independently of its size (Garfin and Heerdt 2001).

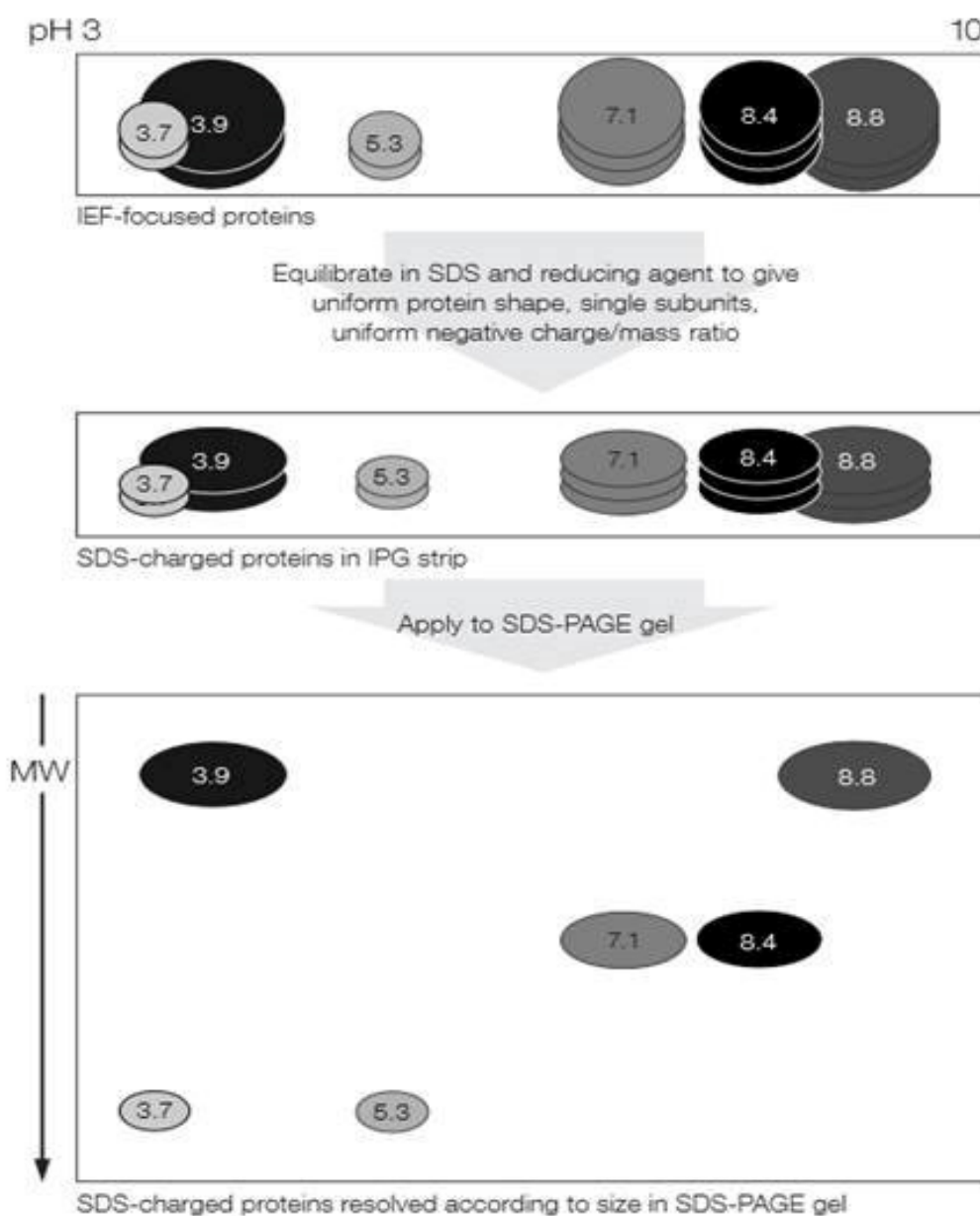


### *Second-dimension separation*

Equilibration is a conditioning step applied to proteins already separated by isoelectric focusing, prior to the second-dimension run. The process reduces disulfide bonds and alkylates the resultant silylthiol groups of the cysteine residues (Goerg et al. 2009).

During that stage, proteins are coated with sodium dodecyl sulphate (SDS) for orthogonal separation (SDS-PAGE), based on their molecular weight. The ability to run many gels at the same time, under the same working conditions, is particularly helpful in performing gel to gel comparisons at later stages (Goerg et al. 1988, 2000) (Figure I.3).

**Figure I.3.** Schematic diagram showing separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after separation by isoelectric focusing (Garfin and Heerdt 2001).



### *Staining and imaging*

In order to visualise proteins in gels, these must be stained. Currently, there exists no ideal universal stain for all types of work, although Coomassie blue and Silver stain are used more often than others. It is noteworthy that metabolically labelled proteins or iodinated proteins are also used (Carroll et al. 2000), as are semi-quantitative protein stains or protein labelling strategies, e.g., difference gel electrophoresis (Alban et al. 2003).

Occasionally, proteins can be detected after transfer to a membrane support by Western blot. For collection of data in digital form, various imaging devices, equipped with the appropriate software to collect, interpret and compare proteomics data, can be used.

### *Protein identification*

There are various types of mass spectrometres, which may be used for protein identification in proteomics studies. Each can achieve that objective in a different way, but the principles of protein identification are similar across the various types and models. Spots of interest are excised from stained gels and, thereafter, proteins are digested with trypsin. Subsequently, the peptides are introduced into the mass spectrometre for ionisation (Gutiérrez et al. 2011).

## **Shotgun or gel-free proteomics**

Shotgun proteomics consists of the analysis of proteomes in their original complex form and allows gel-free differential protein relative abundance analysis of complex protein mixtures to reach suitable power and availability to compete with gel-based methods, following an initial digestion step.

High-resolution liquid chromatography (HPLC), multi-dimensional (ion exchange, reverse-phase or affinity) nano-capillary chromatography, in combination with mass spectrometry, are performed, with the aim to provide massive protein identification results (Mazzucchelli and De Pauw 2013). In HPLC, peptides are first generated through prior enzyme digestion (usually by using trypsin) and molecules are separated in liquid phase. The most commonly employed technique is reverse-phase HPLC separation, in which fractionation of peptides is based on their hydrophobicity, through columns usually C18 or C8. The sample is prepared in aqueous solution and then injected into the column, where the molecules interact with the hydrophobic material (Olver 2011).

Another common technique is strong cation exchange chromatography, which separates peptides based on their net positive charge (Machtejevas et al. 2006, Danielsen et al. 2011). Furthermore, multi-dimensional chromatographic separation is increasingly applied, because it can be automated and it helps highly charged and/or hydrophobic proteins to generate peptides accessible to mass-spectrometry (MS) analysis (Yates et al. 2009). In this technique, ion exchange is mostly used as a first dimension, in order to desalt ion exchanged fractions before MS; reverse-phase chromatography is used for second dimension for further fractionation based on hydrophobicity of proteins, i.e. of samples (Gritti et al. 2007).

Following this step, samples are transferred automatically, for minimizing sample loss, to a mass spectrometre (MS) or a tandem mass spectrometre (MS/MS), which is usually coupled with the HPLC (LC-MS) and introduced for ionisation. When compared to the two-dimensional polyacrylamide gel electrophoresis, shotgun proteomics can be more sensitive, because there is no requirement to recover proteins or peptides from a gel matrix, so it can provide a tremendous larger amount of data supporting as well the accuracy of the results.

## **Mass spectrometry (MS)**

Advancements and commercialisation in mass spectrometry field (MS), as well as the success of the genome projects have concluded in the existence of the proteomic science. Mass spectrometres are particularly sensitive instruments, which can identify peptides in the attomole ( $10^{-18}$ ) to femtomole ( $10^{-15}$ ) range (Moyer et al. 2003, Urban et al. 2010). This sensitivity helps to identify peptides in minute concentrations in small-size samples. Except from sensitivity, important factors that characterise a mass spectrometre is the resolution, the dynamic range and the ability to select and separate ions of interest. There is a great variety of types of spectrometres, which can be used for proteomics studies; however, all of them include three essential components: ionisation source, analyser(s) and detector(s).

### **Ionisation methods**

Ionisation methods are matrix-assisted laser desorption/ionisation (MALDI) (Yates 1998, Peffers et al. 2013), which require mixing of the peptide with an ultraviolet-absorbing molecule and the formation of crystals. Specifically, a laser strike of the crystalline structure results in sublimation of the matrix and ionisation and release of the associated peptides. Surface-enhanced

laser desorption/ionisation (SELDI) (Mach et al. 2010, te Pas et al. 2012) is a variation of MALDI using a target, modified to achieve biochemical affinity with the compound under analysis. The sample and the matrix co-crystallise, as the solvent evaporates. In SELDI, the protein mixture is spotted on a modified surface, which affords a chemical functionality. Some proteins in the sample bind to the surface, whilst others are removed by washing. After washing of the spotted sample, the matrix is applied to the surface and is allowed to crystallise with the sample peptides. Binding onto the SELDI surface acts as the first-separation step; the subset of proteins that bind to the surface are easier to analyse.

Electrospray ionisation (Fenn et al. 1989), also widely used in proteomics studies (Restelli et al. 2012, 2013), consists of a technique used in mass spectrometry for production of charged ions from proteins, peptides and other non-volatile macromolecules. The method is termed to be a 'soft' ionisation method, because fragmentation during the process is minimal and involves use of high voltage to charge the liquid containing the analyte, which forces the liquid through a small capillary tube to form an 'aerosol mist' of charged droplets. The solvent evaporates, leaving the multiple-charged molecular ions progressing towards the mass analyser for detection. As it is liquid-based, electrospray ionisation is easily coupled to front-end fractionation by liquid chromatography (Danielsen et al. 2011, Kocaturk et al. 2013).

## Mass analysers

Ions produced during ionisation are separated according to their mass. A peptide's identity is expressed as a ratio of mass divided by the charge of the peptide ( $m/z$ ), through the application of electromagnetic fields in a mass analyser. Main types of mass analysers are as follows.

A quadrupole mass analyser consists of four parallel rods with fixed direct constant voltage and alternating radio frequency (RF) field potentials applied to them. Ions produced at the source of the instrument are focused and passed along the middle of the quadrupoles, their motion depending on electric fields, so that only ions of a particular  $m/z$  would have a stable trajectory, thus going through to the detector. This type of operation is termed 'mass-selective stability' (Hommerson et al. 2011, Xu et al. 2011).

Ion cyclotron resonance is related to movement of ions in a magnetic field. It is used for accelerating ions in a cyclotron and for measuring the masses of ionised analytes in mass spectrometry, particularly with Fourier transform ion cyclotron resonance mass spectrometers (FTMS). FTMSs measure mass by detecting the image current produced by ions cyclotroning in the presence of a magnetic field. The detectors have fixed positions in space, which measure the

electrical signal of ions passing near them and producing a periodic signal. Since frequency of an ion's cycling is determined by its  $m/z$ , this can be de-convoluted by performing a Fourier transform on the signal. FTMSs have a high sensitivity (since each ion is 'counted' more than one time) and a high resolution, therefore an increased precision (te Pas et al. 2012).

Ion trap mass analysers combine electric and magnetic fields, which capture ions in a region of a vacuum system or tube. They are similar to the quadrupole mass analysers in that radio frequency-voltages are applied to produce an oscillating ion trajectory. The term 'ion trap' is derived from the fact that the fields are applied so that ions of all  $m/z$  are initially trapped, oscillating within the mass analyser. Mass analysis is achieved by means of 'mass-selective instability', because all ions (bar those with the selected  $m/z$  ratio) are retained in the fields of the mass analyser. At the end, a mass spectrum is obtained by changing electrode voltages, with the aim to eject ions from the trap. Advantages of the ion-trap mass spectrometres include the smaller size of the instruments, as well as their ability to trap and accumulate ions to increase the signal-to-noise ratio of a measurement (Hsu et al. 2013).

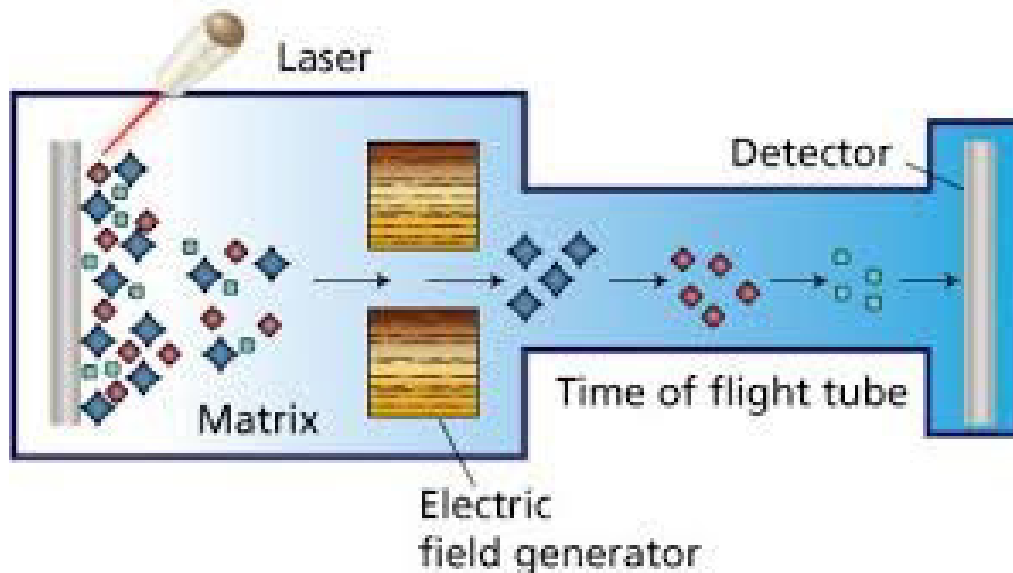
The most commonly used hybrid types of ion traps in biological sciences can be separated principally into the following two types: quadrupolar ion traps (e.g., Paul traps, linear Paul traps) (March and Todd 1995, Hogenboom et al. 2009) and Fourier transform (FT) ion traps (e.g., orbitraps and FT penning traps) (Scigelova et al. 2011). A quadrupolar ion trap is based on the same principles as the quadrupole mass analyser, but the ions are trapped and sequentially ejected. A linear quadrupolar trap is similar to that, but traps ions in a two-dimensional quadrupole field, instead of a three-dimensional quadrupole field as in a three-dimension quadrupole ion trap (Prentice and McLuckey 2012). In FTMS, instead of measuring deflection of ions with a detector, as discussed above, the ions are injected into a penning trap (a static electric/magnetic ion trap), where they effectively form part of a circuit (Patrie et al. 2004).

Tandem mass spectrometres use the separation of ions, according to their  $m/z$ , as a preparative tool to isolate an ion with a specific  $m/z$  for further analysis. This further analysis is carried out by fragmenting the mass-selected ion by determining the  $m/z$  of the fragment ions in a second stage of mass analysis. The term 'tandem mass spectrometry' reflects the fact that two stages of mass analysis are used in a single experiment. In this respect, more complex mass spectrometres have been built, which implies that tandem spectrometres are instruments with over one analyser, thus for use in structural and/or sequencing studies. Two, three or four analysers (not necessarily all being of the same type) have nowadays been incorporated into commercially-available tandem instruments (hybrid instruments).

The main two categories of tandem mass spectrometres are: (i) tandem-in-space mass spectrometres and (ii) tandem-in-time mass spectrometres. The first type includes quadrupole-quadrupole and quadrupole-time-of-flight geometries. The second type includes mainly ion traps and orbitraps (Fourier transform) mass spectrometres (Patrie et al. 2004, Prentice and McLuckey 2012).

The operating principles of time-of-flight (TOF) mass analysers are simple (Figure I.4). The analyser employs an electric field of high voltage to accelerate the ions, which are all given the same amount of initial kinetic energy. Following acceleration, the ions enter a field-free area, where they travel at a velocity inversely proportional to their  $m/z$ . Consequently, ions with small  $m/z$  travel more rapidly than ones with great  $m/z$ . Time required for the ions to travel the entire length of the field-free zone is measured and used to calculate ion velocity, hence  $m/z$  of each ion (Wollnik 1993). A hybrid type of TOF, which is used often in proteomics is quadrupole/time-of-flight mass spectrometres (Q-TOF) (Morris et al. 1996, Shevchenko et al. 1997, Danielsen et al. 2011). In these, a 'parent ion' is selected in a quadrupole mass filter Q1 and broken up in a collision cell within an RF-only ion guide; this may be a hexapole or a second quadrupole Q2 (Shevchenko et al. 1997). 'Daughter ions' enter a reflecting TOF spectrometre, which examines the complete mass spectrum with no scanning (parallel detection). Whereas the TOF mass analyser utilises a vacuum free-fall chamber, in this case ions are electrically accelerated (i.e., not in free-fall). The uniform potential applied then, translates to acceleration causing a specific mass with a specific charge to travel with different velocities inside a TOF flight tube (Guilhaus 1995). Masses are separated and analysed based on the 'ion drift' principle, i.e., heavier ions reaching the detector later than lighter ions with the same charge. These devices have high sensitivity, high resolving power and high mass accuracy, so they have advantages over the triple-quadrupole configuration for high performance measurements of biomolecules.

**Figure I.4.** Schematic illustration of the function of a matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) mass spectrometre (Sigma 2016).



## Detector

The detector is the final component of a mass spectrometre. The detector monitors and amplifies the ion current and transmits the signal to the data system for recording as mass spectra. There, the  $m/z$  value of each ion is plotted against its intensity, in order to show number of components in the sample, molecular mass of each component and relative abundance of the various components in the sample. Type of detector is supplied to suit the type of analyser; the more common ones are the photomultiplier, the electron multiplier and the micro-channel plate detectors.

Finally, identification of a protein is based on measurement of the multiple peptides originating from that. For example, after a mass spectrometre has determined the  $m/z$  for the peptides from a gel spot, this information will be matched to a protein database. A successful protein match is based on number of peptides matched to the protein and the accuracy of the matches.



## Bioinformatics

'Bioinformatics tools' for proteomics, also termed 'proteome informatics tools', include an array of various applications, from simple tools for comparison of amino acid composition of proteins to sophisticated software for large-scale determination of protein structure. There are bioinformatics tools specific for two-dimensional electrophoresis analysis, for liquid chromatography/mass spectrometry analysis and/or for protein identification by peptide mass fingerprinting, peptide fragment fingerprinting (PFF) or *de novo* sequencing analysis (Palagi et al. 2006).

### Two-dimensional gel electrophoresis image analysis

In general, two-dimensional electrophoresis gel image analysis softwares have the same basic operations and functionalities necessary to carry out a complete gel study, which should end up with highlighting differentially expressed spots in populations of gels. Dowsey et al. (2003) have reviewed in detail relevant techniques. Apart from fundamental visualisation properties, the major functions of software systems for two-dimensional electrophoresis gel image analysis are (i) detection and quantification of protein spots on the gels, (ii) matching of corresponding spots across the gels and (iii) localisation of significant protein expression changes. Various other features (e.g., data management, database integration) may be included in there.

Matching gel images is also a critical process, whether based on previous detection of spots (Pleissner et al. 1999) or on intensity of the regions before detection of spots (Smilansky 2001). That depends on similarity of the spatial distribution of spots from a gel image shot as a reference image and another gel image, which then may vary according to experimental gel running conditions and gel scanning. These issues should be discarded, while keeping only the changes in protein expression.

Various authors (e.g., Rogers et al. 2003) have evaluated various relevant software packages (PDQuest, Melanie 3 [named ImageMaster 2D Platinum since ver. 5], Phoretix, Progenesis and Z3 [not supported any more]) for their ability to be used for the above purposes and have concluded that all performed well in all evaluations. Two further comparative studies showed equivalent results in comparing PDQuest *versus* Progenesis (Rosengren et al. 2003), PDQuest *versus* Phoretix (Wheelock and Buckpitt 2005) and Melanie *versus* Z3 (Raman et al. 2002). Nevertheless, above results are not very conclusive in terms of selecting one as the best software, as all have important features, e.g., management of mass spectrometric, 2-DE or other

relevant data, that need to be considered by a use in an integrated way, powerful comparative analysis with multiple statistical values to increase reliability in the results, *etc.*

## LC-MS image analysis

Another proteomics workflow combines separation of proteins and peptides by liquid chromatography followed by direct analysis by mass spectrometry. This image analysis promises applications in differential proteome analysis by comparing several proteome sets, detecting significant quantitative differences and discovering specific proteins (Berger et al. 2002, Palmblad et al. 2002).

Various operations and functionalities are necessary to carry out a complete LC-MS study. As part of those, filtering removes peaks with weakest intensities (e.g., 'background noise') or high spikes constant in time (e.g., 'chemical noise' including column contaminants). That way, the complexity of spectra is reduced, which facilitates peak detection. Peak detection involves looking for monoisotopic peaks (also termed 'de-isotoping procedure') and determining states of the ion charge. Finally, isotopic peaks of the same corresponding mass value are clustered into one single peak signal. By using this procedure, peaks of interest can be selected from the enormous quantity of data (Hernández-Castellano et al. 2015).

Proteomics applications of relevant software programs are limited at the moment and lack all necessary and useful functions. Examples of such softwares include SpecArray (Li et al. 2005) and Mzmine (Katajamaa and Oresic 2005).

## Peptide mass fingerprinting analysis and peptide fragment fingerprinting analysis

A protein or a mixture of proteins is digested by using appropriate enzymes and the resulting peptide masses are measured, producing a spectrum, also termed 'peptide mass fingerprinting' (PMF). Peptides can also be isolated and fragmented within the mass spectrometre (e.g., by collisional activation), leading to spectra, a process termed 'peptide fragment fingerprinting' (PFF).

In peptide mass fingerprinting, the test spectrum is compared with theoretical ones computed from protein sequences stored in databases or digested *in silico* (virtually) by using the same cleavage specificity of the protease employed in the experiment. The procedure counts overlapping masses between test and theoretical spectra, leading to 'similarity scores' for each

candidate protein. These are then arranged according to their scores. Top-ranked protein or proteins (the latter in case of homologies in the database or of several proteins in the test spectrum) is/are considered as the identification of the spectrum. The key step of the procedure lies in the scoring function. Many factors must be taken into account to produce a robust score, including dissimilarities in the peak positions due to internal or calibration errors or modified amino acids, expected peak intensities, noise, contaminant or missing peaks. A variety of different scoring schemes have been implemented in various algorithms and some of them have been integrated into available software (Palagi et al. 2006).

Various software for peptide mass fingerprinting have been developed and evaluated (Gras and Muller 2001, Hernández et al. 2006). Among these, PeptideSearch (Mann and Wilm 1994) and PepFrag (Fenyo et al. 1998) use a simple score based on number of common masses between test and reference spectra. Pappin et al. (1993) designed a scoring function/algorithm termed 'MOWSE', which accounts for non-uniform distribution of protein and peptide molecular weights in databases. Other score schemes are exploited in MS-Fit (Clauser et al. 1999), MASCOT (Perkins et al. 1999), ProFound (Zhang and Chait 2000) and Aldente (Gasteiger et al. 2005).

PFF analysis is similar to PMF, with the fundamental difference being that correlates peptide spectra with theoretical peptides from a database; theoretical spectra are computed from theoretical peptide sequences and compared to test spectra, in order to find the most similar candidate peptide (Palagi et al. 2006). Mass spectrometre-based identification has advantages over peptide mass fingerprinting; it is possible to work with complex peptide mixtures or to search homologous databases, detailed information about peptide sequence and potential modifications or mutations can be obtained. At the end, all peptides of a given protein have to be confirmed for accurate identification of the protein (Palagi et al. 2006). Consequently, all softwares function in a similar way to those working with peptide mass fingerprinting; this consists of the following workflow: (i) database digestion, (ii) candidate proteins and peptides filtering, (iii) similarity scoring and (iv) result validation. Typically, they allow searching in various databases, e.g., the National Center for Biotechnology Information database (Wheeler et al. 2004) or the UniProt Knowledge base (Swiss-Prot and TrEMBL) (Wu et al. 2006).

Currently, peptide fragment fingerprinting analysis is the most commonly used approach for identifying proteins in complex mixtures.

## *De novo* sequencing analysis

*De novo* sequencing analysis refers to inferring knowledge regarding a peptide sequence independently of any information from a pre-existing protein or from any database. The peptide sequence or part(s) of it are read by measuring distances between peaks in the MS/MS spectrum and searching for distances that correlate either to the mass of a single amino acid (most of their masses are distinct) or a combination of amino acids forming a peptide (Alterovitz et al. 2006). *De novo* sequencing algorithms work in a search space composed of the set of all possible sequences that can be represented by the spectrum, with the only restriction being the arrangement of the peaks (Palagi et al. 2006).

Due to the size required for this search space, *de novo* sequencing analysis methods require spectra of high quality with small fragment errors, an aspect in which they are at a disadvantage compared to peptide mass fingerprinting analysis and peptide fragment fingerprinting analysis.

On the other hand, however, they may detect peptides and proteins with no bias from known databases. If high quality spectra are available, *de novo* sequencing analysis can provide better results over peptide mass fingerprinting analysis and peptide fragment fingerprinting analysis when searching genomic databases subjected to sequencing errors, when searching databases composed of homologous sequences in the case of cross-species identification and when analysing spectra from a mutated proteins or protein variants (Frank et al. 2005).

*De novo* sequencing analysis can be used for cross-species identification (Liska and Shevchenko 2003) or in the peptide fragment fingerprinting analysis to generate partial sequence information, in order to filter candidate peptides prior to identification (Frank et al. 2005).

Various relevant softwares have been developed, e.g., GutenTAg, DeNovoX, Spectrum Mill, PEAKS, Lutfisk, AUDENS, PepNovo and Sequit! (Ma et al. 2003, Tabb et al. 2003, Frank and Pevzner 2005, Grossmann et al. 2005).

## **B. APPLIED PROTEOMICS IN VETERINARY MEDICINE**

### **Applied proteomics in small ruminant health management**

#### **Proteomics studies in healthy sheep or goats**

##### *Body fluids (bar of the reproductive system)*

Studies have described the blood serum proteome of sheep (Chiaradia, Avellini et al. 2012) and the blood plasma proteome of goats (Anagnostopoulos et al. 2013). Leak et al. (2004) have described the lymph proteome of healthy sheep and found that its overall protein map was similar to that of blood plasma; nevertheless, some proteins were found to be present only in lymph (e.g., glial fibrillary astrocyte acidic protein, neutrophil cytosol factor-1), along with the enrichment of some haemostatic factors (fibrinogen proteins and derived peptides). The proteome of urine of sheep or goats has not been described.

Other studies have described the proteome of saliva of sheep or goats; this can be used as index of nutritional status (Lamy and Mau 2012, Mau 2012), of feeding behaviour (Lamy et al. 2009) or of health status (Kuruvilla and Cristobal 2012, de Sousa-Pereira et al. 2013) of sheep or goats. Morphew et al. (2007) studied the proteome of bile of healthy sheep. Finally, proteomics analysis of the mucin-containing fraction of gastric mucus revealed presence of gastric mucin (Muc5ac) and several co-purifying proteins, including intelectin-2 (Pemberton et al. 2011).

Initially, the requirements and difficulties of using ovine cerebrospinal fluid for proteomics studies have been described by Chen et al. (2006). Later, other researchers have reported that differential expression of albumin has been detected in the cerebrospinal fluid of sheep according to their age, with the concentration of the protein increasing progressively (Chen et al. 2010). More recently, Teixeira-Gomes et al. (2015) have indicated that proteomics indicators in the cerebrospinal fluid of ewes were affected by photoperiod; these authors detected different proteins present in cerebrospinal fluid samples collected in days with long or short photoperiod. Finally, Shamsi et al. (2011) have studied the proteome of tears.

##### *Body tissues (bar of the reproductive system)*

There are only a few studies, which have investigated proteomics in tissues of sheep or goats. Proteomics studies have been reported with reference to evaluation of muscle tissues with

a view to understanding skeletal muscle function (Hamelin et al. 2007) or development (Hegarty et al. 2006) or to improving meat quality (McDonagh et al. 2006). Other studies involved gastrointestinal tissue samples, e.g., rumen epithelium (Bondzio et al. 2011) or jejunum (Schönhusen et al. 2010), with a view to understanding nutritional physiology. The methodologies have also been used to clarify and understand the loci of the major histocompatibility system in sheep (Ballingall et al. 2008). Heart tissue samples from sheep fetuses, as well as newborn or growing lambs, have been examined by proteomics techniques, aiming to understand developmental physiology of the cardiac muscle (Buroker et al. 2008). Proteomics techniques have also been used for examination of sheep lung (Kycko and Reichert 2008), goat liver (Jiang et al. 2014) or goat adipose tissue (Restelli et al. 2014) samples. Finally, there are also relevant studies, where proteomics analysis of tissues of healthy sheep has been performed with a view to use the findings for primary application in subsequent studies in humans, e.g., in erythrocytes (Scambi et al. 2010) or in tissues of ophthalmic (Tao and Julian 2014) or dental (Mrozik et al. 2010) origin.

Extensive studies have been performed in samples of wool or hair, aiming to lead to improvement of the quality of these products. Flanagan et al. (2002), Plowman (2003), Clerens et al. (2010) and Plowman et al. (2012) have produced proteome maps of the wool of various sheep breeds. In later studies, the effects of various management practices in the quality of wool have been investigated by Almeida et al. (2014) and Liu, Li et al. (2014). Goat fibre quality has been evaluated using proteomics techniques by Thomas et al. (2012), whilst Seki et al. (2011) investigated expression of keratin in hair and Yang, Cai et al. (2015) studied development of hair. Finally, evaluation of the quality of leather produced by sheep or goat skin by use of proteomics techniques has been proposed by Choudhury et al. (2006).

#### *Fluids and tissues of the reproductive system*

Relevant works have referred to the study of maturation of spermatozoa at the epididymis of rams (Gatti et al. 2005), the study of epididymal fluid and other ram reproductive tract fluids (Souza et al. 2012), the study of ram seminal plasma (Bergeron et al. 2005, Cardozo et al. 2006, Druart et al. 2013, Soleilhavoup et al. 2014, Rickard et al. 2015, Rocha et al. 2015), the evaluation of sperm membrane in buck semen (van Tilburg et al. 2015), the characterisation of uterine luminal proteins in ewes at the initial stage of pregnancy (Koch et al. 2010), the identification of peptides in amniotic fluid (Viviers et al. 2015), the description of intra-uterine growth trajectory of ovine embryos (Serchi et al. 2013) and the identification of proteins in the cervicovaginal fluid of ewes during parturition (Young et al. 2007, Dellios et al. 2010).

Further, the study of milk and milk production has received attention and has been greatly facilitated by proteomics technologies. Relevant studies include the identification of peptides in ovine (Signorelli et al. 2012, Ha et al. 2015) or caprine (Tay and Gam 2011, Anagnostopoulos et al. 2014, Cunsolo et al. 2015) milk or in ovine colostrum (Hernández-Castellano, Almeida, Castro et al. 2014, Hernández-Castellano, Almeida, Ventosa et al. 2014, Scumaci et al. 2015), as well as the profiling of milk fat globule proteome (ewes: Pisanu et al. 2011, Spertino et al. 2012; does: Cebo et al. 2010) and the detailed description of caseins in caprine milk (Roncada et al. 2002, Hinz et al. 2012). Finally, other studies researched the protein intake after colostrum ingestion by newborn lambs, by evaluating the protein profile in the blood plasma (Hernández-Castellano et al. 2015); this can contribute in confirming the degree of protection of lambs.

Proteomics in genital system tissues have been applied by Wang, Wang, Hu et al. (2013) and Al-Gubory et al. (2014), in samples of ovine uterine endothelium, by Ramadoss and Magness (2011) in samples of ovine uterine artery and by Jia et al. (2014) in samples of ovine ovaries. Finally, mammary tissue samples have been studied by Signorelli et al. (2012) (ewes) and Lérias et al. (2014) (does).

## Proteomics studies in diseases of sheep or goats

### *Mammary diseases*

In ewes and does, there are only few studies presenting proteomics work in mastitis. These refer to improving accuracy and early detection of mastitis, to identification of virulence factors of causative agents and to studying host-pathogen interactions and host immune response.

Chiaradia et al. (2013), in milk samples from field cases of ovine mastitis, used 2-D PAGE coupled with MALDI-TOF MS and/or nano LC ESI-LIT MS/MS analysis (ESI: electrospray ionisation, LIT: linear ion trap). By using the above methods, they have identified various proteins, which were expressed differentially in milk whey, hence were potential biomarkers for the diagnosis of the disease. They also suggested that, in ewes, protein detection might be better (in terms of accuracy, especially at the early stage of the disease) diagnostic method than somatic cell counting. Olumee-Shabon and Boehmer (2013) have identified a phosphopeptide (tetraphosphopeptide) of the milk protein alpha(s2)-casein, which was absent in cases of mastitis in goats and thus could possibly be used as marker for diagnosis of the disease. More recently, Pisanu et al. (2015), after experimental infection of ewes and application of advanced proteomics technologies (proteomic analysis by LTQ-Orbitrap Velos mass spectrometry), identified in total 287

proteins with upregulation in cases of mastitis, which were potential biomarkers for diagnostic purposes.

Le Maréchal et al. (2009) employed 2-D PAGE, immunoblotting and spot sequencing to describe immunogenic proteins of *Staphylococcus aureus* in blood serum collected from experimental mastitis. This might have helped to identify and define the organism's core seroproteome (i.e., a pool of proteins common to all *S. aureus* strains) and accessory seroproteome (i.e., a pool of immuno-detected proteins that vary with the *S. aureus* strain and the ewe infected). Bacterial surface proteins were resolved (by using sodium dodecyl sulphate-PAGE [SDS-PAGE]) and transferred on a membrane and, then, incubated with different serum pools from sheep with experimentally induced *S. aureus* mastitis, that way identifying highly immunoreactive staphylococcal proteins. It was found that proteins from bacterial cells harvested from culture media during the growth phase were of higher immunogenicity than proteins harvested from the same cells during the stationary phase. These findings could explain the results of previous *in vivo* experimental work, which had indicated that intramammary challenge of ewes with staphylococcal cultures after an incubation period of five hours (i.e., obtained during the growth phase of the challenge strain) resulted consistently to mastitis (Fthenakis and Jones 1990). Moreover, serological proteome analysis (SERPA) of ewes with mastitis led to the identification of 89 immunogenic proteins in *S. aureus*; of these, 74 constituted the organism's core seroproteome (Le Maréchal et al. 2011).

Specifically for *S. aureus* ovine mastitis, Seyffert et al. (2012) indicated N-acetylmuramyl-L-alanine amidase as an immunoreactive protein of significance in the host reaction to infection. The results have a potential significance in the development of immunological tools against *S. aureus* mastitis.

Proteomics studies have indicated no increased presence of blood serum albumin in the milk of goats after intramammary lipopolysaccharide infusion (Olumee-Shabon et al. 2013). The findings indicate that in goats no breakdown of blood-milk barrier occurs in such circumstances, which is contrasting with the respective situation in cows (Hogarth et al. 2004, Boehmer et al. 2008).

Addis et al. (2011) have investigated the proteomic profiles of milk fat globule membrane proteins in ewes infected with *Mycoplasma agalactiae*. This enabled detection of enrichment of several proteins involved in inflammation, chemotaxis of immune cells and antimicrobial defences, e.g., cathelicidins or calprotectin (S100-A8/S100-A9), in infected animals, suggesting the consistent involvement of mammary epithelial cells in the innate immune response to pathogens. The authors concluded that pro-inflammatory proteins (S100 proteins, cathelicidins) were



produced by the mammary epithelial cells and participated in the defence against invading pathogens. Then, the authors showed an ability of mammary epithelial cells to produce and release various defensive molecules, by studying the cathelicidin and calprotectin subunit S100-A9 on mammary tissues. In another study, where *Streptococcus uberis* was used as the challenge model, similar results were described (Addis et al. 2013). Although *S. uberis* is of little significance as a mammary pathogen in ewes, the work was useful as a model for bovine mastitis and highlighted the non-specific defence reaction of the ovine mammary gland. The same research group, by applying the *S. uberis*-infection model provided new hypotheses regarding the defence role of neutrophil extracellular traps in the mammary gland and associated them with mammary epithelial cells (Pisanu et al. 2015).

### *Other diseases*

#### Systemic or multi-organ diseases

Chemonges et al. (2014) established a framework for understanding susceptibility of sheep to infection, specifically to bacterial endotoxin, by using proteogenomics techniques. Identification of disease-related acute phase proteins and micro-ribonucleic acids (miRNAs) provided a foundation for further studies, to understand involvement of endotoxin-related acute phase proteins and miRNAs, to determine the cause of dysregulation in challenge with lipopolysaccharide and to develop new assays based on detecting acute phase proteins and/or miRNAs. Liu, Lin et al. (2008) used proteomics methodologies (2-D PAGE coupled with MALDI-TOF MS and Q-TOF MS/MS) and identified the spore coat-associated protein as a strong immunogenetic factor of *Bacillus anthracis*, the causative agent of anthrax in sheep or goats; moreover, they found that an experimental vaccine containing that protein was able to induce antibody production against the organism in mice.

Proteomics investigations of *Corynebacterium pseudotuberculosis* isolates recovered from lymph nodes of sheep revealed in total >1350 proteins in the strains. The findings indicated a protein abundance in the organism, which might play a role in the diversity of pathogenic actions that it shows (Rees et al. 2015).

#### Diseases of the genital system

Wagner et al. (2002) used 2-D PAGE and matrix assisted laser desorption/ionisation mass spectrometry (MALDI MS) and detected 883 protein spots in *Brucella melitensis* isolates. They presented a complete proteome map of the organism, which can be associated with virulence factors and immunogenicity, for use as a reference. Eschenbrenner et al. (2002, 2006) used 2-D PAGE and peptide mass fingerprinting and described the proteome of the Rev 1 vaccinal strain

and the 16M pathogenic strain; they identified that expression of an immunogenic bacterial outer membrane protein, of proteins utilised in iron acquisition and of proteins playing a role in sugar binding, lipid degradation and amino acid binding was modified in Rev 1. Finally, Mavromati (2012) used proteomics methodologies (LC MS), establishing differences in various blood serum proteins between sheep infected or not with *B. melitensis*, when aiming to monitor progress of vaccination for controlling the infection and Wareth et al. (2015) studied the immunogenic proteins of *B. melitensis* in the blood of infected sheep.

Proteomics techniques (2-D immunoblots) have been employed in samples from ewes experimentally infected with *Chlamydophila (Chlamydia) abortus*. Samples from allantoic fluid, amniotic fluid, ewe blood serum and foetal blood serum have been tested and results indicated that antibodies in pregnant ewes increased soon after infection and were followed, after the 40th day post-challenge, by foetal antibodies (Marques et al. 2011).

The immunogens of *Campylobacter jejuni* clone SA for sheep have been identified by studying infective strains of the organism, as well as blood samples from infected sheep. In total, 26 immunogenic proteins were detected, of which 8 were cytoplasmic proteins, 2 were cytoplasmic membrane proteins, 11 were periplasmic proteins, 3 were outer membrane proteins and 2 were extracellular proteins (Wu et al. 2014).

#### Diseases of the alimentary system

Hughes et al. (2007), by using 2-D PAGE and MALDI-TOF, identified ten proteins with upregulated expression in strains of *Mycobacterium paratuberculosis* subsp. *avium* isolated from the ileum of sheep with paratuberculosis. Later, Hughes et al. (2008) reported the immunogenic effect of these proteins and proposed their inclusion in candidate vaccines. Hughes et al. (2012) then presented the proteomic differences of *M. paratuberculosis* subsp. *avium* type I/III and type II strains, which are the two phenotypic classes of the organism. The same researchers also proposed the use of proteins differentially present in the blood serum as a potential tool for accurate diagnosis of subclinical paratuberculosis in sheep (Hughes et al. 2013). Similar findings have been reported by Zhong et al. (2011), who used chromatographic techniques and tandem mass spectrometry and have identified two biomarkers (transthyretin and  $\alpha$ -haemoglobin) in the blood serum of sheep exposed to *M. paratuberculosis* subsp. *avium*.

Research into pathogenesis and control of gastrointestinal nematodes has also benefited by use of proteomics methodologies. Identification of proteins, which are expressed by nematodes (e.g., *Haemonchus* galactose-containing glycoprotein complex, cysteine proteases) (Knox et al. 2001), can provide antigens for inclusion in novel antiparasitic vaccines. Kiel et al. (2007) have used proteomics and identified immunogenic proteins in *Trichostrongylus colubriformis* infective

larvae, with the final aim to prevent establishment and development of these larvae in the host (sheep), which might lead to control of the infection. Athanasiadou and Huntley (2008) have referred to changes of the proteome in the intestinal mucosa of sheep during nematode infections, e.g., the upregulation of intelectin (among others), which is part of the innate host defence mechanisms and plays a role in exclusion of *Teladorsagia circumcincta*; these findings may relate to the cellular activity in the gut during nematode infections. Goldfinch et al. (2008) have identified changes in the proteome of gastric lymph (specifically, in the regulation of gelsolin,  $\alpha$ -1  $\beta$  glycoprotein, haemopexin), which have occurred as the result of *T. circumcincta* infection and which can contribute to elucidation of the local response in the digestive tract of infected animals. IgA and IgE activity against *T. circumcincta* was investigated in a sheep flock; it was found that IgE activity, which was found to be associated with decreased faecal egg counts of the parasite, was under genetic control and moderately heritable (Murphy et al. 2010). Nagaraj et al. (2012) have identified differences in protein expression in the abomasal surface between sheep resistant to *Haemonchus contortus* infection and those susceptible to the nematode, which can be used in the understanding of host-pathogen interactions and in the sustainable control of the infection. Abomasal tissue samples of sheep infected or not with *T. circumcincta*, were evaluated for differential expression of proteins that may play a role in host protection and inhibition of parasite establishment (Pemberton et al. 2012). Finally, Wang, Yuan et al. (2014) studied the immunomodulation induced by recombinant galectins of *H. contortus* (rHco-gal-m/f) on goat mononuclear cells and identified 16 differentially expressed proteins involved in stimulus response, biological regulation and localisation; these authors also indicated that the activation of vascular endothelial growth factor pathway, free radical producing pathway and ubiquitin-proteasome pathway was inhibited following exposure to rHco-gal-m/f, while that of toll-like receptors pathway and caspase pathway was promoted.

Proteomics methodologies have also been employed in the study of trematode infections. Jefferies et al. (2001) and Morphew et al. (2007, 2011) have identified cathepsin L proteases in the excretory-secretory products of *Fasciola hepatica* in the bile duct of hosts. Chemale et al. (2010) studied the differences in the proteome of triclabendazole-resistant and triclabendazole-susceptible strains of *F. hepatica*, by means of which molecular mechanisms related to resistance development by the parasite were revealed.

#### Diseases of the respiratory system

Ayalew et al. (2010) employed 2-D PAGE immunoblot analysis and MALDI-TOF MS and LC MS/MS and have identified 55 proteins with immunogenic properties on the outer membrane of *Mannheimia haemolytica*, a pathogen of major importance in sheep and goats (Bell 2008); among

these, there were proteins involved in cell structure, in transport/virulence, in the general metabolism of the organism, in cell process, in translation and in DNA replication, regulation, transcription and virulence. They suggested that, as a consequence of their immunogenic role, in the future, these proteins might be employed in production of novel vaccines (Ayalew et al. 2010), findings which were subsequently used for producing experimental vaccines for sheep (Ayalew et al. 2011). Subsequent work, by means of LC MS/MS or SDS-PAGE and immunoblot analysis, identified the immunogenic properties of the outer membrane vesicles of this organism (Ayalew et al. 2013, Roier et al. 2013), which were also used in the production of experimental vaccines against the infection.

Boehmer et al. (2011) employed proteomics methodologies (nano LC MS/MS) in samples of bronchoalveolar lavage from animals with experimental *M. haemolytica* infection. They identified various proteins uniquely present in the lavage, which included antimicrobial peptides (cathelicidin-1, cathelicidin-4), complement factors, acute-phase proteins (haptoglobin, inter- $\alpha$ -trypsin inhibitor heavy chain-4), protease inhibitors and molecules involved in oxidation-reduction. Finally, Nanduri et al. (2005) indicated that administration of antimicrobial agents resulted in the reduction of the expression of *M. haemolytica* leucotoxin, which might explain the protective effect of metaphylactic antibiotic administration in outbreaks of respiratory infections in lambs (Mavrogianni and Fthenakis 2005).

Kycko and Reichert (2008, 2012) have applied 2-D PAGE and LC MS/MS in lung tissue samples from confirmed cases (by means of immunohistochemistry) of ovine respiratory adenomatosis, caused by *Jaagsiekte Sheep RetroVirus*. They identified over-expression of cytokeratin 19, aldolase A and mangan superoxide dismutase and proposed their use in the accurate diagnosis of the disease.

#### Diseases of the nervous system

Proteomics has been used in order to elucidate the molecular pathogenesis of prion diseases of ruminants, with the aim to contribute to their early diagnosis by identifying relevant biomarkers (Molina et al. 2010, Ma and Li 2012) or to establish control strategies (Chich et al. 2007). Other studies have contributed in the elucidation of the structure of prion protein, by using proteinase K for protein digestion in the process of molecule identification (Petrotschenko et al. 2012).

Samples of brains from heterozygous scrapie-infected sheep were examined by means of proteomics, to identify polymorphisms of *PrP* in the pathogenesis of transmissible encephalitis (Morel et al. 2007). Petrakis et al. (2009) have identified various host molecules with high affinity for *PrP<sup>Sc</sup>* from brain tissues of sheep. In a study performed in rats with experimental scrapie, Giorgi

et al. (2009) have identified ferritin, calcium/calmodulin-dependent protein kinase a type II, apolipoprotein E and tubulin as the major components associated with *PrP*<sup>27-30</sup>, as well as trace amounts of actin, cofilin, heat shock protein HSP 90- $\alpha$ , the  $\gamma$  subunit of the T-complex protein 1, glyceraldehyde 3-phosphate dehydrogenase, histones and keratins. Some of these proteins (tubulin and ferritin) are known to bind *PrP* (Kim et al. 2007, Dong et al. 2008); other proteins (calcium/calmodulin-dependent protein kinase a type II, heat shock protein HSP 90- $\alpha$ ) may be associated with *PrP*<sup>TSE</sup> fibrils during disease. In infections of squirrel monkeys challenged with mouse *PrP*<sup>Sc</sup> derived from sheep scrapie, apolipoprotein E and actin have also been observed in association with *PrP*<sup>TSE</sup> (Nakamura et al. 2000). Apolipoprotein E co-localisation occurred in moderately mature lesions in prion diseases, where it may play a role in the aggregation of *PrP*<sup>Sc</sup> after a conformational change from cellular *PrP* isoform to *PrP*<sup>Sc</sup>. Biomarkers for diagnosis of scrapie in sheep, which has been identified by using proteomics from the brain of mice with experimental disease, can be Na<sup>+</sup>/K<sup>+</sup> ATPase (Graham et al. 2011), which enhanced the efficiency of disease-specific conversion of recombinant *PrP*, suggesting that it may act as a molecular cofactor. Further, amyloid A has also been found differentially to show upregulation and presence in the blood serum (Meling et al. 2013) and PrP(C) has been found in increased concentrations in the urine (Andrievskaia et al. 2008) of affected sheep, hence they may be used in the diagnosis of the disease. Finally, 9-aminoacridine compounds have been found to reduce the *PrP*<sup>Sc</sup> burden, thus providing new ideas for potential drugs against the disease (Phuan et al. 2007). Molina et al. (2010) have proposed the use of transthyretin monomer in the early diagnosis of the infection, when the aetiological agent spreads from the lymphoid organs to the brain.

In the topic of microbial diseases of the nervous system, Schar et al. (2010) have revealed that mutants of *Listeria monocytogenes*, another important zoonotic pathogen of small ruminants, at *pycA* (the gene encoding for pyruvate carboxylase) cannot replicate intracellularly and are thus attenuated in mice challenge trials.

#### Diseases of other systems

Myers et al. (2007), *in vitro* studies, used proteomics methodologies and identified eight immunogenic proteins among the virulence factors and cell surface proteins of *Dichelobacter nodosus*, the causal agent of footrot (Winter 2011); three of these were extracellular proteases, which contributed significantly in the virulence of the organism and could be used in candidate vaccines.

Acute renal failure has led to presence of increased quantities of calbindin-D28k, retinol-binding protein 4 and CD1d proteins in the affected sheep (Palviainen et al. 2012).

## Applied proteomics in health management of other farm animal species

### Proteomics studies in cattle

#### *Healthy cattle*

Proteomics investigations in healthy cattle have referred to the study of the proteome of various body fluids. Samples examined included blood (e.g., Wait et al. 2002, Yang et al. 2010, Marco-Ramell et al. 2012, Ma, Bu et al. 2015), lymph (e.g., Bianchi et al. 2007), urine (e.g., Bathla et al. 2015, Xu et al. 2015), saliva (e.g., Lamy and Mau 2012, Mau 2012, de Sousa-Pereira et al. 2013), tears (e.g., Shamsi et al. 2011), cerebrospinal fluid (e.g., Brenn et al. 2009), bronchoalveolar lavage (e.g., Mitchell et al. 2008), seminal plasma (e.g., Druart et al. 2013, Boe-Hansen et al. 2015, Rego et al. 2015), uterine fluid (e.g., Faulkner et al. 2013, Forde et al. 2014), milk (e.g., Yang et al. 2010, Bislev, Deutsch et al. 2012, Lu et al. 2014, Ha et al. 2015, Zhang, Boeren, Hageman et al. 2015, Zhang, Boeren, van Hooijdonk et al. 2015) and colostrum (e.g., Reinhardt and Lipollis 2008, Nissen et al. 2012). Further, proteomics studies have been performed in a variety of tissue samples from healthy animals, including brain, liver, kidney, mammary gland, muscle and adipose tissue (e.g., D'Ambrosio et al. 2005, Kuhla et al. 2007, Sauerwein et al. 2014).

Proteomics has also been employed to elucidate various reproductive processes and to facilitate reproductive control, e.g., to study spermatogenesis (Thundathil et al. 2012), to investigate fertility of bulls (Gaviraghi et al. 2010), to improve sperm sexing (Chen et al. 2012), to investigate fertility of cows (review by Kurpinska et al. 2014), to profile the milk fat globule proteome (Bianchi et al. 2009, Affolter et al. 2010, Yang, Zheng et al. 2015a,b), to provide details about milk synthesis and production of milk lipids (Beddek et al. 2008, Peng et al. 2008, Lu et al. 2013) and the glucose and lactose metabolism during lactation (Rawson et al. 2012).

#### *Mastitis*

Mastitis, as a significant disease of cows, has received wide attention in application of proteomics, by means of which an amount of knowledge has been generated regarding the diagnosis of subclinical mastitis, with promising results.

By employing 2-D PAGE, mass spectrometry and/or shotgun proteomics, various proteins present in blood serum (Kim et al. 2011, Alonso-Fauste et al. 2012, Turk, Piras et al. 2012), milk (Danielsen et al. 2010, Larsen et al. 2010, Smolenski et al. 2011, 2014, Alonso-Fauste et al. 2012, Hinz et al. 2012, Roncada et al. 2012, Wheeler et al. 2012, Mansor et al. 2013, Reinhardt et al.

2013, Safi et al. 2013, Bian et al. 2014, Guerrero et al. 2015) or mammary tissue (Yang et al. 2009, 2014, Bislev, Kusebauch et al. 2012, Huang, Luo et al. 2014, Zhao, Yang et al. 2015) from cows with mastitis have been identified. Moreover, proteomics technologies have been employed in the analysis of neutrophils (Lippolis and Reinhardt 2005, Liu et al. 2012), in the study of milk fat globule (Smolenski et al. 2014) and in the evaluation of molecular mechanisms of the host response, e.g. N-linked glycosylation (Yang et al. 2014).

Detection of such proteins can be of use in elucidating pathogenetic pathways, e.g., pathogen-leucocyte interactions, pathogen recognition, direct antimicrobial function, modulation of inflammatory signalling and expression of immunoglobulins in the milk. Further, use of proteomics techniques could improve accurate diagnosis of mastitis at the early stages, which would subsequently support effective treatment of the disease.

Finally, proteomics studies of mastitis-causing organisms, e.g., of *Escherichia coli* strains (Hinz et al. 2012, Mansor et al. 2013, Lippolis et al. 2014, Blum et al. 2015), of *S. aureus* strains (Taverna et al. 2007, Mansor et al. 2013, Peton et al. 2014), of *S. epidermidis* strains (Savijoki et al. 2012) and of *S. uberis* (Poutanen et al. 2009), have identified potential targets for vaccine development and have disclosed mechanisms employed by invading bacteria to survive in the host environment (Boehmer 2011). Proteomics studies have also suggested that *S. aureus* and *S. uberis* can increase dramatically biofilm growth in mammary tissues, by increasing mutagenesis and adaptation, in order to resist to antibiotics (Varmanen et al. 2012).

#### Other diseases

Proteomics has been applied in the study of various other diseases of cattle. Among these are anaplasmosis (Molad et al. 2013), bovine viral diarrhoea (Lee, Nanduri et al. 2009), brucellosis (Kim et al. 2014, Wang, Zhang and Zhao 2014), endotoxaemia (Kocaturk et al. 2013), foot-rot (Sun, Zhang et al. 2013), ketosis (Xu and Wang 2008, Xu et al. 2015), neosporosis (Fritz et al. 2012), respiratory infections caused by *M. haemolytica* (Boehmer et al. 2011) or *Mycoplasma mycoides* subsp. *mycoides* (Weldearegay et al. 2016), ruminal acidosis (Jiang et al. 2014), schistosomiasis (Pérez-Sánchez et al. 2006), theileriosis (Witschi et al. 2013) and transmissible spongiform encephalopathy (Simon et al. 2008, Nomura et al. 2009).

#### Proteomics studies in pigs

Proteomics investigations in healthy pigs have referred to study of the proteome of body fluids: blood (e.g., Roncada et al. 2007, Miller et al. 2009, Grubbs et al. 2015, Ozgo et al. 2015),

urine (e.g., Havanapan and Thongboonkerd 2009), saliva (e.g., Gutiérrez et al. 2011, 2013, Lamy and Mau 2012, Fuentes-Rubio et al. 2014), cerebrospinal fluid (e.g., Rosenling et al. 2009), seminal plasma (e.g., Druart et al. 2013), follicular fluid (e.g., Fahiminiya et al. 2012), milk (e.g., Silva et al. 2010, Ogawa et al. 2014) and colostrum (e.g., Wu et al. 2010, Danielsen et al. 2011, Ogawa et al. 2014). Further, proteomics studies have been performed in a variety of tissue samples from healthy animals, including brain, the choroid plexus, lungs, heart, mononuclear cells, intestine, pancreas, kidney and uterus (e.g., Danielsen et al. 2006, 2007, Ramirez-Boo et al. 2006, Huang et al. 2008, Thanos et al. 2009, Chae et al. 2011, Jeon et al. 2013, Gilpin et al. 2014, Kooij et al. 2014, Zhang, Dai et al. 2014, Kubo et al. 2015) to establish normal proteomes of the respective tissues or to elucidate various processes, e.g., the foetal implantation (Jalali et al. 2015) or development (Chae et al. 2011, 2012).

Various diseases of pigs have also been studied by use of proteomics. These include *E. coli* intestinal infections (Wu et al. 2015), foot-and-mouth disease (Liu et al. 2011), hepatitis E (Rogée et al. 2015), *Porcine circovirus* infection (Ramirez-Boo et al. 2011, Cheng et al. 2012, Liu, Bai et al. 2014), respiratory infection by *Actinobacillus pleuropneumoniae* (Kahlisch et al. 2009), *Salmonella* intestinal infections (Martins et al. 2012, Arce et al. 2014, Collado-Romero et al. 2015, Marco-Ramell et al. 2015), swine fever (Sun et al. 2010, Sun, Shi et al. 2011) and swine influenza (Zhu et al. 2012). Further, this animal species has also been used as a model for the study of various mechanisms, conditions and disorders of humans (Bassols et al. 2014).

## Proteomics studies in other farm animal species – selective review

### *Studies in buffaloes*

Proteomics studies in buffaloes include investigations into the proteome of muscular tissue (Kiran et al. 2015, 2016), of saliva (Muthukumar, Rajkumar, Rajesh et al. 2014) and of ocular fluid (Varma et al. 2015). Studies related to reproduction have referred to identification of proteins in the cervicovaginal fluid (Muthukumar, Rajkumar, Karthikeyan et al. 2014), the proteome of caruncles and the chorioamnion (Balestrieri et al. 2013) and blood serum proteins as indicators of pregnancy (Balhara et al. 2014); other works have identified the proteome of testis (Huang, Fu et al. 2014), as well as proteins in semen potentially associated with high- or low-fertility animals (Huang, Fu et al. 2015).

The proteome of mammary tissue has been reported by Jena et al. (2015). Colostrum proteins have been reported by Bhanu et al. (2016), milk whey proteins have been reported by



Yang et al. (2013), whilst Ren et al. (2013) have described differences in milk proteins between breeds of buffaloes.

Studies relevant to diseases include investigations of virulence factors of *Pasteurella multocida* isolates from healthy animals or animals with respiratory disease (Kamran et al. 2014).

#### *Studies in camels*

Proteomics studies in camels (in most cases dromedary camels) have reported proteins in blood serum (Malik et al. 2013), urine (Alhaider et al. 2012), tears (Chen, Shamsi et al. 2011, Shamsi et al. 2011), seminal plasma (Druart et al. 2013), milk whey (Yang et al. 2013) and milk fat globule membrane (Saadaoui et al. 2013) of those animals. Alyamani et al. (2011) have used proteomics for identification of proteins as biomarkers for diagnosis of paratuberculosis in dromedary camels.

#### *Studies in cervids*

Proteomics studies in cervids include primarily research in the development and regeneration process of antlers of red deer, which is the only known organ in mammals that undergoes regeneration. Relevant studies include work in producing proteome maps of antlers (Park et al. 2004), in evaluating various technical approaches for protein identification (Gao et al. 2010) and in investigating signaling pathways of antler stem cells (Li 2012). Other proteomics works refer to detection of angiotensin I inhibitors in blood plasma of red deer (Liu et al. 2010) and to identification of proteome in the sperm membrane of sika deer (Kawase et al. 2015).

Studies relevant to diseases include investigations of virulence factors of a *Pseudocowpox virus* strain from reindeer and of *Rhipicephalus microplus* ticks from white-tailed deer (Popara et al. 2013).

#### *Studies in South America camelids*

The few proteomics studies in South American camelids refer to reports of proteins in seminal plasma of alpacas (Druart et al. 2013) and milk of llamas (Saadaoui et al. 2014).

#### *Studies in chickens*

In chickens, the proteome of blood (Huang et al. 2006, Tyler et al. 2008, Gilbert et al. 2011, Li et al. 2012), semen (Labas et al. 2015) and genital tract fluids (Sun, Xu and Yang 2013) have been described. Little work has been performed with proteomic studies in eggs, due to the abundance of a few major proteins therein, which make the process difficult; nevertheless Guerin-

Dubiard et al. (2006) have performed proteomics studies and identified 16 proteins in the egg albumen.

Proteomics technologies have been used to produce a proteome map of chicken gonadal primordial germ cells, used to better understand germ cell development (Han et al. 2005). Intestinal mucosa tissue samples have also been tested with proteomics (Luo et al. 2013). Further, skeletal muscle samples from broilers have been examined using 2D-PAGE and MALDI-TOF (Doherty et al. 2004), which may support the industry to understand processes taking place during muscle development in broiler chickens.

Challenge of broiler chickens with various gastrointestinal pathogens has resulted in upregulation of actin and actin-related proteins in the intestine; however, no systemic changes, e.g., increase of  $\alpha$ -1-acid-glycoprotein, were recorded (O'Reilly et al. 2013), findings which indicate that small-extent, localised changes might be leading to the adverse effects observed in such diseases. Similar results have been found after challenge with *Salmonella enteritidis*, in which case specific upregulation of mucin was evident (Revajova et al. 2012). Moreover, eight proteins have been identified in blood serum samples of chickens infected with enteropathogenic *E. coli* strains (Tyler et al. 2008). Various strains of *S. enterica* serovar *gallinarum* have been tested under *in vitro* conditions, aiming to identify proteins for vaccine production (Kang et al. 2012). Finally, after challenge with *Eimeria* species, namely *E. acervulina*, *E. maxima*, *E. tenella*, three different proteins: malate dehydrogenase 2, NADH dehydrogenase 1 alpha subcomplex 9 and ATP synthase, have been identified for potential use as biomarkers for early diagnosis of the infection (Gilbert et al. 2011).

Detailed proteomics studies of *P. multocida* (Boyce et al. 2006, Tabatabai 2008) have identified various proteins, which may act as virulence determinants of the organism. Other studies have 51 proteins with differential expression levels, of which 25 were recorded with upregulation and 26 with downregulation during *Avian Reovirus* infection (Chen, Wu et al. 2014). In tracheal tissues inoculated with *Infectious Bronchitis Virus*, *Newcastle Disease Virus* or *Avian Influenza Virus* subtype H9, there were differing protein expression changes which would contribute to elucidation of pathogenetic mechanisms prevalent in the respective infections (Sun et al. 2014). Deng et al. (2014) have reported challenge of chickens with *Newcastle Disease Virus* and evidence regarding the proteomic response of peripheral blood mononuclear cells.

Proteomics methodologies have been applied extensively in the study of Marek disease (Haq et al. 2010, 2013, Kunec 2013). Changes in relevant proteomes have been reported by Thanthrige-Don et al. (2009, 2010) in the spleen, Lu et al. (2010) in the Fabricius bursa, Hu et al. (2012) in the thymus samples, Chen, Li et al. (2011) in the liver and Chen, Hu et al. (2014) in

cutaneous skin tissues after infection with *Marek Disease Virus*. Further, Kumar et al. (2012) have investigated molecular mechanisms of neoplastic transformation in Marek disease, as a model for studying human lymphomas, and found that NF-kappa B is a key protein complex in neoplastic transformation of CD30-expressing lymphocytes *in vivo* during the disease.

Wu et al. (2012) have characterised the cellular proteome changes of the bursa of Fabricius in bursal disease and have identified 54 altered cell proteins; of these, 12 were seen with notable upregulation and 42 with considerable downregulation.

#### *Studies in other poultry species*

Little work has been performed in other poultry species. Proteomics techniques have been used for studying anatine ovarian follicles (Han et al. 2016), anatine hepatocytes (Zhao et al. 2010) and anatine liver tissue samples (Zheng et al. 2012). Mechanisms, at the level of hepatic proteins, underlying liver steatosis in waterfowl 'mule ducks' (i.e., common duck female birds [*Anas platyrhynchos*] inseminated by semen from Muscovy drakes [*Cairina moschata*]) have also been reported (Bax et al. 2012). Mule ducks have been also used in studying fatty liver with the scope of characterising quality parameters of the product (Awde et al. 2015) and cooking losses (Theron et al. 2011, Awde et al. 2014), in order to identifying the mechanisms implicated in production of foie gras (François et al. 2014), specifically, relationships between liver protein compositions and liver quality phenotypes (e.g., liver weight, melting rate, protein content on crude or dry matter).

Proteomics studies of egg white proteins from two poultry species, specifically quail (*Coturnix coturnix*) and duck (*Anas platyrhynchos*), were performed to compare findings with respective work in chicken. The objective was to provide insight into advantages involved in application on egg white proteins from various sources, in order to list benefits for the food industry (Hu et al. 2016).

#### *Studies in aquatic organisms*

Proteomics technologies have been applied to investigate physiology, developmental biology and impact of contaminants in various fish model organisms, e.g., zebrafish (*Danio rerio*), as well as in farmed species, mainly salmonids and cyprinids. However, lack of previous genetic information on most of the species has led to drawbacks for more widespread application of the various proteomic technologies currently available.

Proteomics investigations have been performed in the gill of zebrafish for physiological and biomarker discovery studies (De Souza et al. 2009), in muscles of gilthead sea bream (*Sparus*

*aurata*) (Schiavone et al. 2008) and adipose tissue of rainbow trout (*Oncorhynchus mykiss*) (Weil et al. 2009) for identification of proteins and in kidneys of African catfish (*Clarias gariepinus*) for protein profiling (Robak et al. 2015).

Studies have been performed to investigate proteins in the blood serum of healthy aquatic organisms, as well as possible alterations during stress conditions (Wu et al. 2004: pond loach [*Misgurnus anguillicaudatus*], Brunt et al. 2008: rainbow trout, Liu, Alfonso et al. 2008: Atlantic cod [*Gadus morhua*], Kumar et al. 2009: tilapia [*Oreochromis mossambicus*]). In crucian carps (*Carassius carassius*), stress effects, specifically hypoxia and anoxia, have been studied by investigating changes in protein expression in the brain (Smith et al. 2009). In rainbow trouts, effects of starvation have been studied by investigating changes in protein expression in the liver (Martin et al. 2001).

Relevant studies have been employed for detection of proteins and peptides, with the aim to detect biomarkers for identification / diagnosis of stress or diseases in various aquatic organisms (Rodrigues et al. 2012). These include: Atlantic cod (Karlsen et al. 2012), Atlantic salmon (*Salmo salar*) (Liu, Alfonso et al. 2008, Braceland et al. 2013, Provan et al. 2013, Valdenegro-Vega et al. 2014), common carp (*Cyprinus carpio*) (Eyckmans et al. 2012), European eel (*Anguilla anguilla*) (Roland et al. 2013), gibel carp (*Carassius gibelio*) (Eyckmans et al. 2012), gilthead sea bream (Isani et al. 2011, Schrama et al. 2013), rainbow trout (Brunt et al. 2008, Eyckmans et al. 2012, Bergh et al. 2013, Long et al. 2015), spotted knifejaw (*Oplegnathus punctatus*) (Shuang et al. 2013), tilapia (Jiang et al. 2008, Kumar et al. 2009, Ma, Yang et al. 2015, Zhao, Han et al. 2015), turbot (*Scophthalmus maximus*) (Zhang et al. 2013), yellow stingrays (*Urobatis jamaicensis*) (Marancik et al. 2013) and zebrafish (Lü et al. 2014). Proteomics have also been employed to identify potential virulence factors of pathogens infecting aquatic organisms, e.g., of *Cryptocaryon irritans* (Mai et al. 2015), *Edwardsiella tarda* (Lv et al. 2013, Song et al. 2013, Wang, Wang, Yang et al. 2013, Guo et al. 2014, Buján et al. 2015, Xie et al. 2015), *Streptococcus iniae* (Zhang, Zhang and Sun 2014) and *Vibrio harveyi* (Jia et al. 2013).

## **Applied proteomics in canine and feline medicine**

### **Proteomics studies in healthy dogs or cats**

General studies have aimed at mapping blood serum proteome in dogs (Presslmayer 2002, Atherton, Braceland, Harvie et al. 2013) or horses (Miller et al. 2004) by using two-

dimensional polyacrylamide gel electrophoresis, as well as at correlating the canine proteomes with genetic markers in dogs; e.g., in Portuguese Water Dogs an abundance of retinol-binding protein 4 in blood has been associated with the adiponectin gene, which is involved in regulation of glucose and fatty acid metabolism (Senechal and Kussmann 2011). Atherton, Braceland, Fontaine et al. (2013) have employed one- or two-dimensional polyacrylamide gel electrophoresis and mass spectrometry and have identified a range of proteins in the serum proteome, which were stratified in the following globulin subclasses: albumin (albumin, apolipoprotein A1, serum albumin precursor),  $\alpha$ 1 (albumin, apolipoprotein A1, kininogen-1 isoform 2, serpin peptidase inhibitor-clade A-member 1 precursor, vitamin D binding protein),  $\alpha$ 2 (apolipoprotein A1),  $\beta$ 1 (complement C3, complement C4, haemopexin),  $\beta$ 2 (apolipoprotein A1, complement C3, complement C4, immunoglobulin heavy chains, immunoglobulin k-like polypeptide, serotransferrin isoform 1, an uncharacterised protein) and  $\gamma$  (immunoglobulin heavy chains, immunoglobulin k-like polypeptide, an uncharacterised protein). Very recently, a database, which includes all proteins that have been identified in tissues of domestic dogs (*Canis lupus familiaris*), in health or disease, named 'CanisOme' has been released (Fernandes et al. 2016); the database reunites information on 549 proteins, associated with 63 dog diseases and 33 dog breeds.

Brandt et al. (2014) have identified >500 proteins in normal canine urine samples; some of these had been previously identified in human urine samples as well and have been associated with various diseases of renal or extrarenal involvement. Studies of the follicular fluid in female dogs have been also performed and have resulted in the identification of 21 proteins, some of which have an established role in the ovarian physiology (Fahiminiya et al. 2010, 2012). Saliva peptides in dogs have been studied by de Sousa-Pereira et al. (2013). Nakamura et al. (2012), using 2-DE and MALDI-TOF, have produced a reference map of cerebrospinal fluid of healthy dogs. In cats, proteomics have been used to identify biomarkers in urine (Jepson et al. 2013), as well as properties of semen of male cats (Vernocchi et al. 2014) and interactions between spermatozoa and ovum at the zona pellucida level during the process of fertilisation (Stetson et al. 2015).

Further, studies performed entirely *in vitro* have employed proteomics in order to study or modify animal pathogens, with a view to producing vaccines for the respective diseases, e.g., Shionoiri et al. (2015) have worked in the inactivation of *Feline calicivirus*.

## Proteomics studies in canine or feline diseases

### *Leishmaniosis*

Proteomics may be employed in supporting an early and accurate diagnosis of leishmaniosis. Britti et al. (2010) have reported that various proteins (serum amyloid A, apolipoprotein E, apolipoprotein C-II, probable phospholipid-transporting ATPase, Ig heavy chain V, inter- $\alpha$ -trypsin inhibitor heavy chain H4, IL-2 receptor  $\gamma$ -chain, transient receptor potential cationchannel subfamily V, haptoglobin, probable dimethyladenosine transferase, apolipoprotein A-IV, Ig- $\kappa$  chain, fibrinogen  $\alpha$  chain, fibrinogen  $\beta$  chain, apolipoprotein C-I, nucleoside diphosphate kinase A, alpha-2-macroglobulin, complement C8, transferrin receptor protein 1, DNA-dependent protein kinase catalytic subunit, angiotensinogen, serum paraoxonase/arylesterase 1) were seen with downregulation or upregulation in the blood of dogs with increased titre against *Leishmania* in Indirect Fluorescent Antibody Technique, which is considered the standard diagnostic technique for the infection (Saridomichelakis 2009). Proteomics have also been used for the identification of various proteins of the parasite and the whole exoproteome of the parasite (Braga et al. 2014), which may be involved in the virulence of the organism and the pathogenesis of the disease (e.g., Dea-Ayuela et al. 2006, Daneshvar et al. 2012).

### *Neoplastic diseases*

Proteomics have been repeatedly proposed to be of help in the diagnosis of neoplastic diseases of dogs or cats. Differences in the proteome of tears of dogs, specifically in the level of actin and albumin and in an unidentified protein possibly analogous to human lacryglobulin, have been identified between healthy dogs and dogs with a variety of neoplastic conditions (transmissible venereal tumour, adenocarcinoma, squamous cell carcinoma, sarcomas, lymphomas, mast-cell tumour, myeloma, melanoma) (De Freitas Campos et al. 2008). Thus, it has been proposed that proteomics examination of tears can be of diagnostic value in neoplastic diseases.

Use of proteomics for blood serum examination of dogs with lymphoma (Ratcliffe et al. 2009) indicated an expression pattern of two biomarkers (mass values: 7,041 Da and 74,726 Da), by the use of which samples could be distinguished into ones from dogs with lymphoma or ones from dogs without lymphoma; this might support diagnosis of the disease, as well as prediction of the potential response of the animal to chemotherapy, hence prognosis of the disease. Wilson et al. (2008) emphasized the prospect of glycopeptide profiling by using proteomics methodologies as diagnostic tools in lymphoma cases, given that in such cases the majority of fucosylated

peptides increased. Gaines et al. (2007) identified several biomarker protein peaks in dogs with B-cell lymphoma, which could be used with increased accuracy in the diagnosis of such cases. Use of proteomics for blood serum analysis of cats also identified albumin and  $\beta$ -globulin as potential biomarkers for diagnosis of lymphoma in that species (Gerou-Ferriani et al. 2011).

Identification of specific changes (downregulation of: calretinin, myosin, light chain 2, peroxiredoxin 6, maspin, fibrinogen  $\beta$  chain, vinculin, isocitrate dehydrogenase 1, tropomyosin 1, annexin A5, Rho GTPase activating protein 1; upregulation of: proliferating cell nuclear antigen, ferritin light chain, bombesin, tropomyosin 3, thioredoxin-containing domain C5, adenosine, ornithine aminotransferase, coronin 1A, RAN-binding protein 1, 3-phosphoglycerate dehydrogenase, eukaryotic translation elongation factor 1) in the proteome of tumour-tissue samples from dogs with mammary carcinoma can possibly be used in the prognosis of the metastatic nature of that neoplasia (Klopfleisch et al. 2010); of the above proteins, 19 were found to be analogous to respective proteins used in women for prediction of potential metastasis of breast cancer. Similarly, Klose et al. (2011) have revealed changes in 48 proteins in samples collected at different stages of mammary adenomas, non-metastatic carcinomas or metastatic carcinomas.

Further, four autoantigens (manganese-superoxide dismutase, triose phosphate isomerase, alpha-enolase, phosphoglycerate mutase 1) have been detected to be over-expressed in samples from dogs with mammary tumour (Zamani-Ahmadmahmudi et al. 2014), findings similar to those from related types of neoplasias in humans, thus facilitating the understanding of pathogenesis of the canine disease. Finally, proteomic analysis, with mass spectrometry, of the surface-exposed proteome of a cancerous cell from two canine osteosarcoma cell lines and normal osteoblasts showed that thrombospondin-1, CYR61 and CD44 were present in all cell lines and CD44 was over-expressed in osteosarcoma cell lines (Milovancev et al. 2013).

Alpha-1-acid glycoprotein, apolipoprotein-A1 and apolipoprotein-A1 precursor were differentially expressed in cats with pancreatic carcinoma; this indicated a possibility of the disease occurring simultaneously with acute phase response and alteration of lipid metabolism in animals with pancreatic disease (Meachem et al. 2015).

The value of proteomics in predicting potential metastatic nature of neoplastic cells has also been reported by Chen, Mathias et al. (2011), who identified molecular markers in Madin-Darby Canine Kidney cells indicating epithelial-mesenchymal transition, a process recently implicated in tumour metastasis (Kang and Massague 2004). Changes in the proteome (mortalin, WD repeat domain 1, protein disulfide isomerase A3, T-complex protein 1  $\alpha$  subunit, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase, annexin A6, T-complex protein 1  $\epsilon$  subunit, actin-related protein 3, tryptase  $\alpha/\beta$  1, transferrin, actin  $\beta$ , annexin

A2, serum albumin) of tumour-tissue samples from dogs with mast-cell tumours have been reported by Schlieben et al. (2012), who proposed use of these differences for accurate identification of the disorder.

#### *Cardio-pulmonary diseases*

Sawicki and Jugdutt (2004) subjected dogs to experimental ischaemia-reperfusion cardiac injury, performed proteomics analysis of tissues of the left ventricle of the heart and found a modified proteomics profile, by means of which they identified the key proteins ( $\alpha$  subunit of ATP synthase isoform precursor, creatine kinase M chain, NAD1-isocitrate dehydrogenase, ATP synthase D chain, ventricular myosin) involved in the pathological process. Proteomics helped to identify 44 proteins in the valvular leaflets of Cavalier King Charles Spaniels; among these,  $\alpha$ -tropomyosin and myosin light chain-2 were considered to be useful diagnostic markers of the myxomatous mitral valve degeneration, as they were downregulated in diseased dogs (Han 2009). Lacerda et al. (2009) have also identified 117 differentially expressed proteins in cases of the disorder and found a similar proteomic profile in dogs at the early or the late stage of the disease, concluding that proteome would change at the early stages of the disease, which indicated the extent of the lesions and the possibility of using it diagnostically. Dohke et al. (2006) found that, in cases of congestive heart disease, proteins maintaining morphologic and functional integrity of cardiomyocytes and increasing tolerance against various stress types ( $\alpha$  B crystallin, heat shock protein 27, heat shock protein 20), were increased and increasingly phosphorylated.

Proteomics analysis performed in samples of bronchoalveolar lavage fluid of West Highland White Terriers (Lilja-Maula et al. 2013) helped to reveal mechanisms involved in idiopathic pulmonary fibrosis and to identify biomarkers relevant to its diagnosis. Proteome comparison of bronchoalveolar lavage fluid of dogs with idiopathic pulmonary fibrosis or with non-specific chronic bronchitis revealed similarities in those proteomes, suggesting a similar animal response to those two disease processes. Nevertheless, when proteomes from diseased animals were compared to those of healthy dogs, no specific biomarkers for use in the diagnosis of idiopathic pulmonary fibrosis could be identified.

Hormaeche et al. (2014) have detected differences in protein present in the urine of dogs infected or not with *Dirofilaria immitis*; fibrinogen alpha chain, immunoglobulin gamma heavy chain A and actin 2 homologous to a protein of *Brugia malayi* have been detected only in infected animals. The authors suggested that downregulation of other proteins (found in the urine of healthy dogs) might interfere in the cholesterol metabolism and O<sub>2</sub> transport, in turn affecting condition of parasitised animals and outcome of infection.



### *Disorders of the musculoskeletal system*

In dogs, use of proteomics profiling in cases of Golden Retriever muscular dystrophy identified under-expression of various metabolic proteins, regulated by the transcriptional peroxisome proliferator-activated receptor- $\gamma$  co-activator 1  $\alpha$  (Guevel et al. 2011). The technology has been recently reported to describe differences in protein profile between natural tendons / ligaments and tissue-engineered tendon / ligament constructs (Kharaz et al. 2016), in which it has been found that content of fibrocartilaginous proteins (significant structural proteins) was more abundant in the former; tissue-engineered tendon / ligament constructs contained smaller amounts of extracellular matrix proteins and included a greater proportion of cell-associated proteins than native tissue, corresponding to their decreased collagen and increased DNA content.

### *Other diseases*

Proteomics have also been used in the study of various other diseases of dogs or cats, but in a less detailed way than in the above conditions.

Nakamura et al. (2012) in cerebrospinal fluid samples from dogs with meningoencephalitis identified a neuron-specific enolase, which might be used as a possible biomarker of the disease. Martin-Vaquero et al. (2015) reported over-expression of eight proteins associated with damaged nervous tissue and/or compromised blood-spinal cord barrier.

Administration of antioxidants as a means of control of brain aging in dogs has been found to result in reduced levels in glutamate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, alpha-enolase, neurofilament triplet L protein, glutathione-S-transferase and fascin actin bundling protein in brain tissue (Opil et al. 2008), which confirmed the supporting role of antioxidants in the prevention of age-related neurodegenerative disorders of dogs. Greco et al. (2013) have identified changes in proteomics profiling (e.g., transthyretin) of the cerebrospinal fluid of dogs with degenerative myelopathy, which may be used for improving diagnosis of the disease. Age-related changes in protein expression in the brain have also been identified in cats by Van den Bergh et al. (2003), who, moreover, indicated that, for at least two of these proteins (glial fibrillary acidic protein and collapsing response mediator protein 5), differential expression extended to mRNA level.

Application of proteomics in urine samples from cats may be employed as a prognostic tool for early diagnosis of azotaemia as the result of long-standing kidney disease (Jepson et al. 2013). Use of proteomics has also led to identification of differential expression of urine proteins (neprilysin, napsin A aspartic peptidase) as markers of kidney function and of immunoglobulins

(immunoglobulin G, immunoglobulin A) as markers of *Leptospira* spp. infection in dogs (Nally et al. 2011, Nally 2012).

Kuleš et al. (2014) have used proteomics methods to identify proteins ( $\alpha$ -1-acid glycoprotein, apolipoprotein A-1, antithrombin-III,  $\alpha$ -1-antitrypsin, vitamin D-binding protein, apolipoprotein A-IV, complement C3, serotransferrin, haemopexin,  $\alpha$ -2HS-glycoprotein, haptoglobin,  $\alpha$ -2-antiplasmin, clusterin, leucine-rich- $\alpha$ 2-glycoprotein, inter- $\alpha$ -trypsin inhibitor H4) in the blood serum of dogs with *Babesia* infection, which may be used to study the pathogenesis of the disease, as well as provide novel biomarkers for more accurate diagnosis of the disease. Further to that work, Kuleš et al. (2016) have attempted to identify biomarkers for early detection of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), when associated with *Babesia* infection in dogs, and found that SIRS was characterised by increased paraoxonase 1 and apoA-I and MODS with decreased complement inhibitors (leading to prolonged complement activation) and decreased vitamin-D-binding-protein (as the result of haemolysis and activation of the coagulation cascade occurring during the infection. Adaszek et al. (2014) revealed the presence of a protein fraction of 51-52 kDa only in the blood serum of animals infected with *Babesia*.

In veterinary ophthalmology, Laskowska-Macios et al. (2015) found, by using proteomics methodologies, in kittens with binocular pattern deprivation, a negative regulation of neurite outgrowth, synaptic transmission and clathrin-mediated endocytosis, thereby implicating these processes in normal experience-induced visual cortex maturation.

In veterinary dentistry, proteomics have been employed in the study of canine periodontitis, specifically in samples of gingival crevicular fluid from different stages of the disease (Davis et al. 2016). In total, 406 proteins have been identified, of which 84 were present in all samples, whereas 40 other proteins were found to change significantly in cases to periodontitis associated with gingivitis; haptoglobin has been found to be significantly increased after confirmation with ELISA measurements, possibly indicating a potential biomarker for early diagnosis of the disease.

## **Applied proteomics in medicine of equids**

Horses are important companion animals. Nevertheless, in some parts of the world, equine meat is readily consumed, whilst other species of equids (e.g., donkeys, mules) are farmed (e.g., jennies for milk production) or employed as working animals. In view of the above, proteomics studies in equids are reviewed in a separate section.

## Proteomics studies in horses

### *Healthy horses*

In horses, proteomics have been employed to fully characterise the protein fraction of saliva (de Sousa-Pereira et al. 2013, Jacobsen et al. 2014), cerebrospinal fluid (Broccardo et al. 2014), follicular fluid (Fahiminiya, Labas, Dacheux et al. 2011, Fahiminiya, Labas, Roche et al. 2011) and milk (Miranda et al. 2004, Yang, Zheng et al. 2015a,b) of mares.

### *Diseases*

#### Disorders of the musculoskeletal system

In horses, proteomics have been used extensively for the study of the musculoskeletal system. Desjardin et al. (2012) have described techniques that can be applied specifically in the cartilage and bones of horses, which had been based in two-dimensional electrophoresis, silver staining and liquid chromatography–mass spectrometry. Using these methodologies, Desjardin et al. (2014) identified 53 modulated proteins related to mitochondrial activity, energy and calcium metabolism. Steelman and Chowdhary (2012) identified upregulation of intestinal apolipoprotein APOA-IV in horses with laminitis, which supported an earlier hypothesis that laminar inflammation might be linked to changes in immune regulation in the gastrointestinal tract (Wagner et al. 2003). Application of proteomics in samples of synovial fluid of horses with osteoarthritis or osteochondrosis revealed various proteins that were deregulated during these pathological processes; although some were modified in both disease entities, which indicated occurrence of similar pathways, the increased number of deregulated proteins in osteoarthritis confirmed the inflammatory character of that disease (Chiaradia, Pepe et al. 2012).

Proteomics have been used for the diagnosis of exertional rhabdomyolysis ('tying-up');  $\alpha$  actin, tropomyosin  $\alpha$  chain and creatine kinase M chain were found to abound in muscle biopsy samples from diseased horses; moreover, increased creatine kinase M may indicate that changes in energy distribution in muscle cells may play a role in the pathogenesis of the disease (Bouwman, van Ginneken, Noben et al. 2010). In fact, evaluation of protein changes by using a proteomics approach in blood plasma (Scoppetta et al. 2012) or muscle tissue samples (Bouwman, van Ginneken, van der Kolk et al. 2010) may be used to indicate training levels of racehorses, as such changes are induced by prolonged, aerobic physical exercise. Finally, Peffers et al. (2013) have used proteomics to report changes in the cartilage of horses occurring with aging of the animal and the differentiation from changes that take place during disease.

Finally, Williams et al. (2013) have found that carprofen administration, a non-steroid anti-inflammatory agent, decreased the release of matrix metalloproteinases 1, 3 and 13 and the appearance of a 60 kDa fragment of FN1 in cartilage explant cultures. This explained the therapeutic action of the drug, which is frequently used against the disease.

#### Other disorders

Proteomics have been employed in the study of equine uveitis; their use led to the identification of novel auto-antigens (recoverin, retinaldehyde-binding protein and malate dehydrogenase) (Deeg 2008). In relation to diseases of the reproductive system, a variety of proteins ( $\alpha_1$ -antitrypsin, apolipoprotein A-1, haptoglobin, immunoglobulin G, transferrin, transthyretin) present in the endometrial fluid of mares may have modified expression in cases of streptococcal endometritis. Hence, their detection could be used as a diagnostic method or a tool to predict outcome of treatment (Wolf et al. 2012).

#### Proteomics studies in donkeys or mules

Serum proteome in donkeys and mules has been described by Henze et al. (2011). Peptides in the milk of jennies have also been identified by means of proteomics technologies (Cunsolo et al. 2011, Fasoli et al. 2013, Piovesana et al. 2015), whilst Chianese et al. (2010) have identified specifically caseins in milk ('caseome') of jennies.

### **Applied proteomics in rabbit health management**

Rabbits are traditionally considered and classified as farm animals. Nevertheless, in recent years, a trend has emerged Europe-wide to increasingly handle that species as a companion animal (Edgar and Mullan 2011). Further, rabbits are also frequently employed in biomedical research as animal models for human diseases. In view of the above, proteomics studies in that species are reviewed in a separate section.

Proteomics investigations have referred to studies of the proteome of body fluids, including blood (Li et al. 2011), nasal secretions (Garibotti et al. 1997), tears (Zhou et al. 2007, 2013, Mandal et al. 2011), seminal plasma (Mastrogiacomo et al. 2014) and follicular fluid (Stetson et al. 2012); further, a reference proteome map of the gastrocnemius muscle has been produced, as this muscle is considered to be a model for muscle plasticity (Almeida et al. 2009, 2010), which

may be employed as reference for the proteome of skeletal muscles in these animals (de Almeida 2013, Liu et al. 2013).

Proteomics techniques have been employed in myocardium tissue samples of rabbits, with the aim to study tachycardia-induced heart failure pathways, as rabbits can act usefully as a model for humans. White et al. (2005) have found 53 protein spot differences in reperfused myocardium; most changes were observed to proteins from four functional groups: (i) the sarcomere and cytoskeleton, notably myosin light chain-2 and troponin C, (ii) redox regulation, in particular several components of the NADH ubiquinone oxidoreductase complex, (iii) energy metabolism, encompassing creatine kinase and (d) the stress response. Further, proteomics techniques have been applied in ventricular remodeling post-infarction of rabbits, describing the increase of antioxidative protein expression and enzyme activity after use of ramipril (Chen et al. 2008).

Finally, ceruloplasmin has been considered to be a potentially useful indicator of staphylococcal infections (Georgieva et al. 2012), which are of significance in rabbit production. Mucha et al. (2012) have identified candidate proteins for a vaccine against tularemia (*Francisella tularensis* infection) in those animals, based on the identification of proteins necessary for adhesion of the organism onto endothelial cells.

**CHAPTER II**

**PROTEOMICS ANALYSIS OF BLOOD AND MILK  
OF EWES WITH INDUCED *MANNHEIMIA*  
*HAEMOLYTICA* MAMMARY INFECTION**

## A. INTRODUCTION

Mastitis is a significant disease of sheep. Some time ago, the European Food Safety Authority (2014) has characterised mastitis as the disease of sheep most significantly affecting their welfare. *Mannheimia haemolytica* is a common aetiological agent of mastitis; in ewes suckling lambs, it is the primary pathogen of the disorder, whilst in dairy ewes, it is of lesser significance (Gelasakis et al. 2015).

*Mannheimia haemolytica* is a weakly hemolytic, Gram-negative coccobacillus, with the following complete taxonomy (Euzéby 1997).

- Superkingdom: Bacteria
- Phylum: Proteobacteria
- Class: Gammaproteobacteria
- Order: Pasteurellales
- Family: Pasteurellaceae
- Genus: *Mannheimia*.

There are five named species within the *Mannheimia* genus: *M. haemolytica*, *M. glucosida*, *M. ruminalis*, *M. varigena* and *M. granulomatis*. All species, bar *M. ruminalis*, have been isolated from the upper respiratory tract of healthy ruminants, where they are part of the normal bacterial flora of those animals and can also cause pneumonia. Further, the bacteria have also been isolated occasionally from the intestinal tract of ruminants (Kuhnert and Christensen 2008).

Two species, *M. haemolytica* and *M. glucosida*, have been associated with ovine mastitis (El-Masannat et al. 1991, Watkins and Jones 1992, Arsenault et al. 2008, Omaleki et al. 2010). *M. haemolytica* is the most common cause of mastitis in flocks of meat-type producing systems (El-Masannat et al. 1991, Watkins and Jones 1992, Arsenault et al. 2008, Koop et al. 2010, Omaleki et al. 2010). In contrast, in sheep that are milked, less than 15% of cases of the disease are associated with *M. haemolytica* (Mavrogianni et al. 2007, Gelasakis et al. 2015).

The first reference of *M. haemolytica* as a causal agent of ovine mastitis dates back in 1929 (Leyshon 1929). Nevertheless, detailed work on its significance as a causal agent of mastitis has started in the 1980's (El-Masannat 1987, Watkins 1990). Clinical signs of *M. haemolytica*-associated mastitis include systemic signs (e.g., increase in rectal temperature, increased respiratory rate, anorexia, depression), as well as a variety of mammary signs (e.g., changes in appearance of milk, enlargement and hardening of the mammary gland, pain, subcutaneous

oedema of the udder, discolouration of the udder skin). These become evident within 12 hours of experimental intramammary inoculation with 10 to 2,000 colony forming units (c.f.u.) of the organism (El-Masannat et al. 1991, Fragkou et al. 2008). Pathological findings include petechiae and ecchymotic haemorrhages with neutrophilic infiltration at early stages of the disease, with hyperaemia, haemorrhage, venous thrombosis and necrosis of mammary parenchyma at later stages; the lactiferous ducts become filled with fibrin, milk clots and inflammatory exudates (El-Masannat et al. 1991, Mavrogianni et al. 2005, Fragkou, Mavrogianni et al. 2007).

*M. haemolytica* has a wide range of virulence factors, including a leucotoxin, which is produced by the organism during the logarithmic phase of growth (Zecchinon et al. 2005) and has been characterised in great detail. The ruminant-specific pathogenetic action of the organism could be attributed to the selective cytotoxic action of leucotoxin against ruminant leucocytes (Zecchinon et al. 2005), causing their apoptosis (Atapattu and Czuprynski 2005), thrombocytes and, occasionally, erythrocytes. Less studied virulence factors include sialoglycoprotease, neuraminidase, immunoglobulin peptidase and the capsule (Omaleki et al. 2011). In addition, specific lipopolysaccharides and outer membrane proteins appear to be associated with virulence (Lo 2010). The bacterial capsule also plays a critical role in adherence and invasion of these bacteria (Zecchinon et al. 2005), which can occur as early as within 10 minutes following infection (Vilela et al. 2004). Membrane proteins decrease immune responses of affected hosts and neuraminidase reduces viscosity of mucus supporting bacterial apposition closer to the cell surface.

The organism can be transferred to the teat orifice, and thence to the mammary parenchyma, during sucking by lambs, from the upper respiratory tract of lambs (Scott and Jones 1998, Gougoulis et al. 2008, Fragkou et al. 2011). Factors that predispose infection by this organism include all those that predispose sheep to mastitis (Gelasakis et al. 2015), as well as specific factors that promote the risk of bacterial transfer from lambs to the teat of ewes, e.g., frequent lamb sucking (Gougoulis et al. 2008), increased litter size (Waage and Vatn 2008), chapped teats (which support bacterial accumulation on their skin; Fragkou, Papaioannou et al. 2007) or teat infections by epitheliotropic viruses (which lead to depletion of local teat defences; Mavrogianni et al. 2006).

The teat of the udder plays a protective role against penetration of *M. haemolytica* into the mammary gland, by means of local lymphoid-type defences. These nodular lymphoid accumulations have been characterised and found to contain CD79<sup>+</sup>, CD3<sup>+</sup>,  $\gamma\delta$  T cells, CD68<sup>+</sup> and MHC-II<sup>+</sup> cells. It has been suggested that these lymphoepithelial structures are activated following bacterial invasion and result from recruitment and expansion of antigen-specific lymphocytes *in*



*situ* (Mavrogianni et al. 2005, Fragkou et al. 2010). There appeared to exist a potential genetic background in the presence and function of these lymphoid accumulations, as differences have been identified between breeds of dairy sheep (Fragkou, Skoufos et al. 2007).

As described above (Chapter I, B), there is some published work in proteomics of mastitis. These publications refer to improving accuracy and early detection of mastitis, to identification of virulence factors of causative agents and to studying host-pathogen interactions and host immune response. None of these references have described work related to *M. haemolytica*-mastitis.

Objective of the experiment described in this chapter was the study of differentially expressed proteomes in ewes after experimentally induced *M. haemolytica* mammary infection.

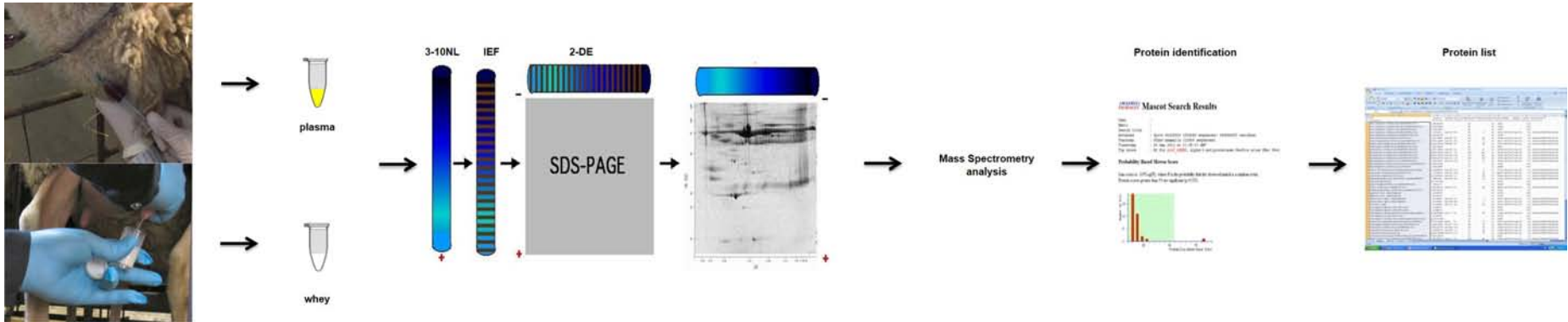
## B. MATERIALS AND METHODS

### Experimental overview

Blood and milk samples from 5 Chios-cross ewes were examined during the study. Animals were inoculated with *Mannheimia haemolytica* into the teat duct, on the 5th day after lambing. Development of mastitis had been confirmed by using clinical, bacteriological, cytological and histological examinations. Blood and milk samples were collected sequentially, before and after challenge, for examination by proteomics technologies: two-dimensional polyacrylamide gel electrophoresis and MALDI-TOF mass spectrometry. Proteins were separated in two-dimensional gels from samples, identified by MALDI-TOF and evaluated for over- or under-expression after inoculation; in some cases, full protein maps have been produced. A graphical overview of the experimental flow is depicted in Figure II.1.

Conditions prescribed by legislation of the European Union in relation to animal experimentation procedures (Council Directive 86/809/EEC) were met during the experiment from which samples were collected; the experiment was carried out under a licence for experimental procedures obtained from the Greek Ministry of Agriculture.

**Figure II.1.** Graphical presentation of the experimental flow in the Experiment ‘Proteomics analysis of blood and milk of ewes with induced *Mannheimia haemolytica* mammary infection’.



## **Inoculation procedure**

A *Mannheimia haemolytica*, strain VSM08L (Mavrogianni et al. 2005, 2006), which has been isolated from the teat duct of a ewe and is of known pathogenicity for the mammary gland of this species, was used for inoculation.

The organism was grown on Columbia blood agar and checked for purity; then it was inoculated into Soy-broth (BioMerieux S.A., Marcy-l'Etoile, France) and incubated aerobically at 37 °C for 5 h. Serial dilutions of the broth culture into sterile phosphate-buffer-saline pH 7.3 (PBS; Dulbecco-Biochrom, Berlin, Germany) were carried out; finally, 0.2 mL of the desired dilution was withdrawn with a syringe. The inoculum contained 1200 to 1250 c.f.u., as estimated by the method of Miles and Misra (1938).

To ensure sterile conditions, on the day before inoculation the hairs of the teats of the experimental ewes were clipped by using fine scissors and the skin of the udder was scrubbed by using chlorhexidine. Ewes were challenged as follows; initially, the teat was disinfected by using iodine povidone solution; then, a sterile plastic fine catheter (Abbocath®; Abbott, Abbott Park, USA) 20 G, 2 mm-long was inserted into one teat; the syringe was attached to the catheter and the bacterial suspension was deposited into the teat duct. The same technique was used to inject 0.2 mL of PBS into the contralateral teat of each ewe. After challenge, lambs were kept away from their dams for 2 hours.

## **Pre- and post-inoculation procedures in the experimental ewes**

### **Clinical examination of the udder**

A general clinical examination of the experimental ewes, with special reference to their udder, was performed before challenge, on the 3rd and 5th days after lambing (D-2, D0, respectively; D0: day of challenge). After challenge, detailed examinations of the animals and collection of samples, were carried out 12 h after challenge (D0+12 h) and thereafter on D1 (1 day after challenge), D2, D3 and D4 (unless a ewe had been removed from the study before that).

## Samplings

On each occasion, when an examination of the udder was performed, sample collection was carried out.

Blood samples were collected into anti-coagulant-treated sterile plastic EDTA-treated tubes. Two tubes were filled from each animal on each occasion. They were mixed by gentle repeated inversions for several seconds to avoid coagulation.

Further, a thorough disinfection was carried out using povidone iodine scrub solution on the teat apex. Milk samples were then obtained. The first two squirts of secretion were drawn onto the palm of the gloved hand of the investigator and examined for the presence of abnormal signs; then, 10 to 15 mL of secretion were carefully collected into a sterile Universal container (for immediate bacteriological and cytological examination) and 25 to 30 mL of secretion into a sterile Falcon container (for storing and subsequent processing by proteomics technologies). Separate samples were collected from each mammary gland of each experimental animal.

Summary of samples collected before and after challenge from the experimental animals is in Table II.i.

**Table II.i.** Summary of blood and milk samples collected before and after challenge and of the various tests performed in these samples.

Days after		Blood samples			Milk samples from infected gland			Milk samples from contralateral gland		
lambling	challenge	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
3	-2		n=1 <sup>a</sup>			n=2 <sup>b</sup>				
5	0	n=5		n=5	n=5		n=5	n=5		n=5
	0 + 12 h	n=5		n=5	n=5	n=1 <sup>c</sup>	n=4	n=5		n=5
6	1	n=5		n=5	n=5		n=5	n=5		n=5
7	2	n=5		n=5	n=5		n=5	n=5		n=5
8	3	n=5		n=5	n=5		n=5	n=5		n=5
9	4	n=2		n=2	n=2		n=2	n=2		n=2

(1) Conventional laboratory tests (haematological examination, bacteriological and cytological examination of milk samples, (2) Proteomics examination for 'full protein map' (one gel from each sample), (3) Proteomics examination for differential protein identification (one gel from each sample).

a: ewe A, b: ewes A and B, c: ewe C.

## Mammary biopsies and tissue sample collection

Mammary parenchyma biopsy samples were collected in three animals three days after challenge (D3) and in two animals four days after challenge (D4). The entire procedure was

performed under sedation and cranial epidural block; opioids were also administered for analgesia (Galatos 2011). All surgical procedures were carried out under strict aseptic conditions. A biopsy of the parenchyma of both mammary glands was performed (Fthenakis and Jones 1990); two cubes of tissue, approximately 1×1×1 cm, were removed from the ventral part of each gland. After obtaining the tissue samples and repairing the wounds, ewes were removed from the experiment.

## **Conventional laboratory examinations in samples from the experimental animals**

### **Haematological examinations**

Haematological examinations were performed in one of the two blood samples obtained from each animal on each sampling occasion. Samples were processed within 30 min after collection.

Initially, blood smears were prepared and kept dry at room temperature. Then, complete haematological examination was performed. Standard haematological tests were carried out by using an automated haematological analyser (Sysmex pocH-100iV Diff; Sysmex Corp., Hyogo, Japan). The following parameters were measured: haematocrit, haemoglobin concentration, mean corpuscular haemoglobin concentration, total leucocyte count and thrombocyte count. Blood smears were evaluated for leucocyte type differentiation.

### **Bacteriological and cytological examinations of milk samples**

Milk samples in Universal containers were plated onto Columbia 5% sheep blood agar using the aseptic technique; the media were incubated aerobically at 37 °C for up to 72 h. Throughout this study, all bacteria isolated were identified by using conventional techniques (Barrow and Feltham 1993, Euzéby 1997).

The California Mastitis Test was carried out in milk samples, as described by Fthenakis (1995) for ewes' milk, by using a reagent (Jorgen Kruuse, Marslev, Denmark). Five degrees of reaction scores ('negative', 'trace', '1', '2', '3'), were recognised, according to the standards of Schalm et al. (1971) and Fthenakis (1995) for ewes' milk. Finally, leucocyte subpopulations were

identified by direct microscopy after Giemsa stain of milk films; in each case, 100 cells were observed and counted.

## **Histological examination of mammary tissue samples**

One of the cubes of tissue from each mammary gland was used for bacteriological examination, by applying procedures as described hereabove. The second tissue sample from each mammary parenchyma was processed for histological examination. Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Haematoxylin and eosin (H&E) standard staining procedures were performed for histopathological study of tissue samples.

## **Preliminary processing of samples for proteomics examination**

### **Processing of samples before storage - Storage**

For proteomics examinations, blood samples and milk samples (from inoculated or non-inoculated glands) collected before or after challenge were processed individually.

The blood sample that was not used in haematological examination, was centrifuged for 15 min at 1000 *g* at 4 °C. The plasma fraction was aspirated and transferred into Eppendorf tubes, which were stored at -80 °C. The process was completed within 60 min after collection. Milk samples in Falcon containers were stored directly at -80 °C within 30 min with no previous processing.

Samples were then transferred packed into dry ice to the laboratory, for processing by proteomics methods. They were further stored at -80 °C until processing.

### **Fractionation of milk samples**

Milk samples were taken out from the deep freeze and left at room temperature to complete thawing. Then, they were centrifuged at 5,000 *g* for 60 min at 4 °C. That way, separation of each sample in three layers was achieved; the middle layer was aspirated and transferred into Eppendorf tubes, for further centrifugation at 16,000 *g* for 30 min at 4 °C. After the second centrifugation, the middle layer was again aspirated and transferred into new Eppendorf tubes, which were stored at -20 °C until further processing.

## **Measurement of protein content of samples by Bradford protein assay**

All samples of plasma extracted from blood samples and of whey extracted from milk samples were initially tested by means of the Bradford protein assay, which provides a colorimetric protein determination method. Samples were taken out of storage and left at room temperature to thawing. They were subsequently maintained under continuous refrigeration. Initially, dilutions of samples were made in distilled water as follows: (i) 1:100, 1:200 and 1:300 for blood plasma samples and (ii) 1:50, 1:100 and 1:200 for milk whey samples.

A 10  $\mu$ L quantity of each of these dilutions was mixed into 2 mL disposable cuvettes (Sarstedt, Nümbrecht, Germany) with 1 mL of a working solution (1 $\times$ ) of Bradford reagent 5 $\times$  concentrate (Bio-Rad, Hercules, USA), which was taken out of refrigeration (4  $^{\circ}$ C), left to room temperature and shaken mildly before usage. The cuvettes were left at room temperature for 5 min, under dark conditions, and, then, placed into a spectrophotometre (SmartSpec Plus Spectrophotometer; Bio-Rad) for total protein amount measurement. A cuvette with distilled water was used as blank sample; no standard sample was used, as the instrument was referenced. The spectrophotometre was set to 595 nm and measurements started from the blank sample and proceeded to test samples.

## **First dimension separation: isoelectric focusing (IEF)**

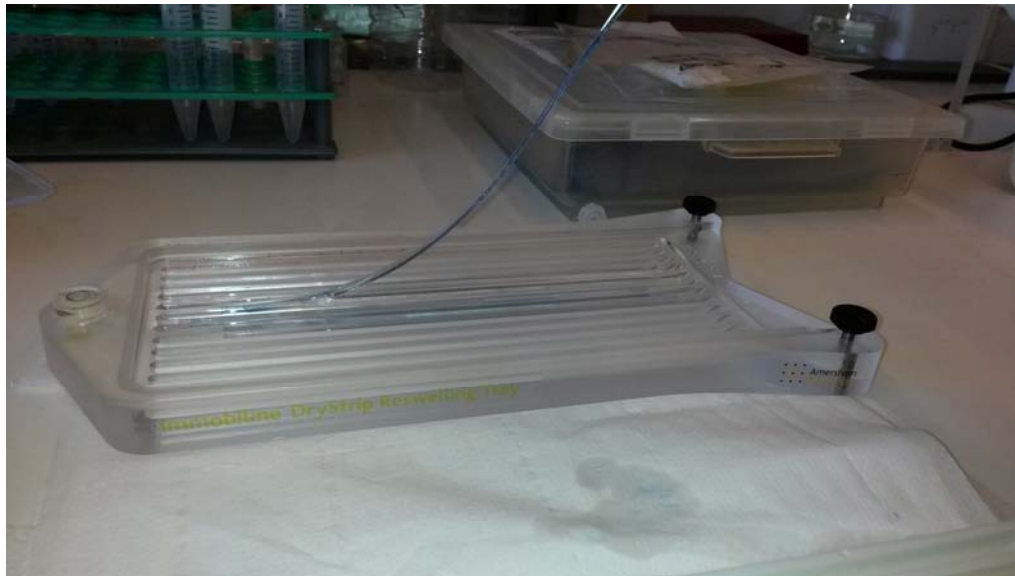
### **Rehydration of IPG strips**

First dimension separation was performed by using 180 mm IPG pH 3-10 Non-Linear strips (Bio-Rad). For rehydration, the strips were immersed into 500  $\mu$ L of rehydration solution (details in Appendix, preparation no. 1) into a tray (Amersham Biosciences, Little Chalfont, United Kingdom), left therein for 11 to 16 hours at room temperature and thereafter removed. The rehydrated IPG strips were dried on absorbing paper (Figure II.2.).



**Figure II.2.** Removal of the IPG strip (blue coloured, due to addition of bromophenol blue) from the tray with the rehydration solution (a) and subsequent drying off on absorbing paper (b).

(a)



(b)



## Electrophoresis

The appropriate volume containing 1 mg protein from each sample (blood plasma or milk whey), which was being maintained under continuous refrigeration, as had been determined by means of the Bradford proteins assay described above, was dissolved into a solution of (i) IEF

sample buffer (details in Appendix, preparation no. 2), (ii) 1 mM phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor (Roche Diagnostics, Basel, Switzerland) and (iii) 0.2% IPG buffer pH 3-10, to a final volume of 250  $\mu$ L. The sample-containing solution was left for 60 min at room temperature and then used for the electrophoresis.

A dried IPG strip was placed into a channel of an electrophoresis tray (or focusing tray) (Bio-Rad), with its side incorporating the gel placed upwards and its poles, (-) (=negative) or (+) (=positive), matching respective poles of the tray. Two electrodes were connected on the tray adjacent to the IPG strip and cups were subsequently pinned at the two edges of the strip and innerly of the electrodes. A quantity of 150  $\mu$ L of the sample-containing solution was added into the cup ('cup loading') at the (-) pole of each tray and the remaining 100  $\mu$ L was added into the cup at the (+) pole of the tray. Each channel was topped with mineral oil (Bio-Rad) and, finally, the tray was transferred into the electrophoresis cell (Protean IEF cell; Bio-Rad) (Figure II.3.).

**Figure II.3.** Placement of dried IPG strips into channels of an electrophoresis tray, with its side incorporating the gel placed upwards and its poles matching respective poles of the tray and connection of two electrodes adjacent to the strips with cups pinned at the two edges of each strip and innerly of the electrodes (a); addition of sample-containing solution into a cup (b, c).

(a)



(b)



(c)



On each electrophoresis tray, up to six channels were filled, thus, up to six blood or milk samples could be tested simultaneously (i.e., up to six strips were processed simultaneously). The following regime was applied; step 1: rapid for 30 min to 250 V - step 2: linear for 15 h to 5,000 V - step 3: rapid for 10 h to 5,000 V - step 4: rapid for 20 h maximum to 500 V, although this step could have been terminated at anytime, to a total focusing of approx. 90,000 Vh; the limit of electric current was 99 mA strip<sup>-1</sup>.

At the end of the procedure, the IPG strips were removed from the tray and excess liquids was dried on absorbing paper. Then, they were placed into plastic Petri dishes, which were sealed

with plastic paraffin film (Parafilm; Bemis, Neenah, USA) and stored at -20 °C up to performing the next step.

## Second dimension separation: SDS-PAGE

### Casting electrophoresis polyacrylamide gels

Electrophoresis non-gradient 12% SDS polyacrylamide gels, with dimensions (length×width×thickness): 180×200×1.5 mm, were hand-cast.

Two ethanol-cleaned glass plates (dimensions: 200×200 mm and 223×200 mm) (Bio-Rad) were placed together, separated by two plastic spacers (thickness: 1.5 mm) between them, located at their edges, forming a 'sandwich', in which the smaller glass (200×200 mm) was placed at the front and the larger (223×200 mm) at the back. Single-screw clamps (Bio-Rad) were tightly applied on the glass sandwich to stabilise it, thus forming a 'cassette', which was then plugged into an assembly station (Bio-Rad). Distilled water was poured into the cassette, in order to check for possible presence of leaks therein, and was subsequently discarded (Figure II.4.).

**Figure II.4.** Two 'cassettes' plugged into respective assembly stations, filled with distilled water, in order to check for possible presence of leaks therein.



Acrylamide solution (details in Appendix, preparation no. 3) was freshly prepared and poured to fill the space in-between the two glass plates, up to a distance of approx. 1.5 mm measured from the upper part of the front glass plate of the cassette. The resulting space was subsequently filled with 50% isobutanol solution (AppliChem, Darmstadt, Germany). Finally, the cassette was covered with plastic wrap and left overnight at room temperature to allow polymerisation of the acrylamide solution.

## Equilibration step

An equilibration stock solution (details in Appendix, preparation no. 4) was taken out of refrigeration and used to prepare two equilibration working solutions (equilibration working solution 1, equilibration working solution 2; details in Appendix, preparations no. 5 and 6, respectively). Petri dishes with IPG strips to be processed were taken out of refrigeration and left to room temperature until defrosting. Subsequently, 10 mL of each of the two equilibration solutions were sequentially added into each Petri dish, which were again left at room temperature, under mildly shaking, for 15 min.

## Application of the IPG strip into the cassette

The plastic wrap was removed from the cassette and the isobutanol solution was washed out with distilled water. The IPG strip to be processed was taken out of the respective Petri dish and its ends were clipped to allow a length of approx. 165 mm. Then, it was placed into the cassette, between the two glass plates, into the space that had been created after washing out the isobutanol solution. The side of the strip incorporating the gel was placed frontward; the (+) pole of the strip was applied in the left part and the (-) pole of the strip was applied in the right part of the cassette. The IPG strip was pushed inwards by using a 1 mm-thick plastic spacer, that way bringing the strip in contact with the polyacrylamide gel. Finally, 1 mL of agarose solution (details in Appendix, preparation no. 7) was poured over the IPG strip, in order to prevent slipping of the strip by stabilising it.

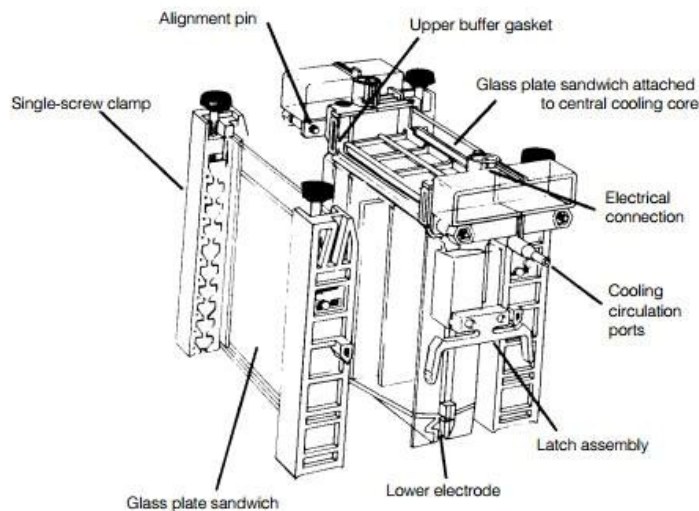
## Electrophoresis

As soon as the agarose solution formed a gel, the cassette was removed from the assembly station and was transferred and attached to the core of the electrophoresis cell (Protean

II xi cell; Bio-Rad). Two cassettes were attached and aligned to the core, which was then inserted into the electrophoresis cell; freshly-made electrophoresis buffer solution (details in Appendix, preparation no. 8) was poured to cover the electrodes of the core. Cooling of the core (Cooler; Amersham Biosciences) was achieved by recirculating water through a cooling bath. The lid of the cell was then applied and the electrodes were plugged into the electric current (Electrophoresis Power Supply, EPS 3510XL; Amersham Biosciences) (Figure II.5.).

**Figure II.5.** Diagram of a cassette attached to the core of an electrophoresis cell (to the right) and of a cassette ready to be attached on it (to the left) (Bio-Rad n.d.) (a); photograph of two electrophoresis cells plugged into the electric current - in the right cell, the blue line is seen during migration from the IPG strip to the other edge of the gel (i.e., from top to bottom) (b).

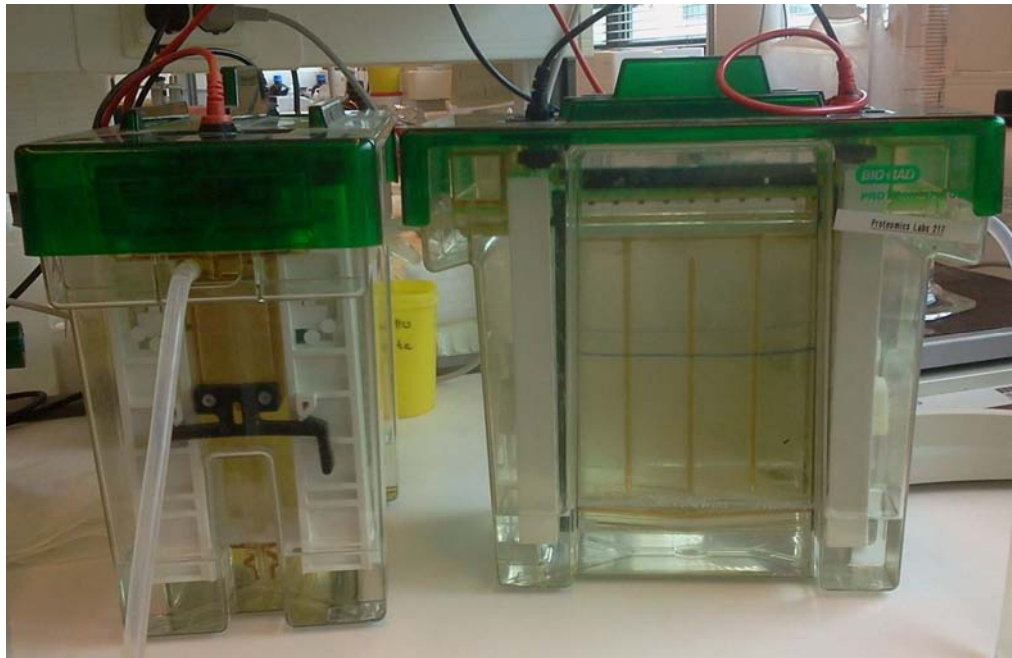
(a)





**Figure II.5.** (continued).

(b)



Electric current was set to 40 mA per gel. In all cases, electrophoresis was performed under the same conditions. The process was considered done when protein separation from the IPG strip had been completed. This was regarded to have been fulfilled, when the blue line (produced as the result of the coomassie blue dye in the agarose solution poured over the IPG strip) was seen to have migrated to the other edge of the gel (Figure II.4.). At that point, the electrodes and the pipe for cooling water were disconnected and the cell was unlied.

### Gel staining

The cassette was opened and the agarose solution gel was excised and removed. Then, very carefully, the polyacrylamide gel was removed and transferred into an electrophoresis gel staining box (Nalgene; Thermo Fisher Scientific, Waltham, USA) containing a fixation solution (details in Appendix, preparation no. 9), wherein it remained for 24 h in room temperature, under mild orbital agitation. On the following day, the fixation solution was discarded and a staining solution (details in Appendix, preparation no. 10) was added in the electrophoresis gel staining box, which remained for 24 h in room temperature under mild orbital agitation.

Then, the staining solution was discarded and the gel was washed with distilled water. Then, the gel was scanned in a calibrated densitometre (GS-800; Bio-Rad) and the image was saved on the equipment hard-disk for performing subsequently appropriate measurements and data analysis. Finally, the gel was inserted into a plastic bag (Sekuroka disposal bag holder; Carl Roth, Karlsruhe, Germany), which was sealed and stored in 4 °C until excision of proteins spots of interest.

Gels and scans of gels were evaluated to identify protein spots of interest.

## **Protein identification**

### **Excision of protein spots of interest**

Protein spots of interest were excised from the gels mechanically or manually.

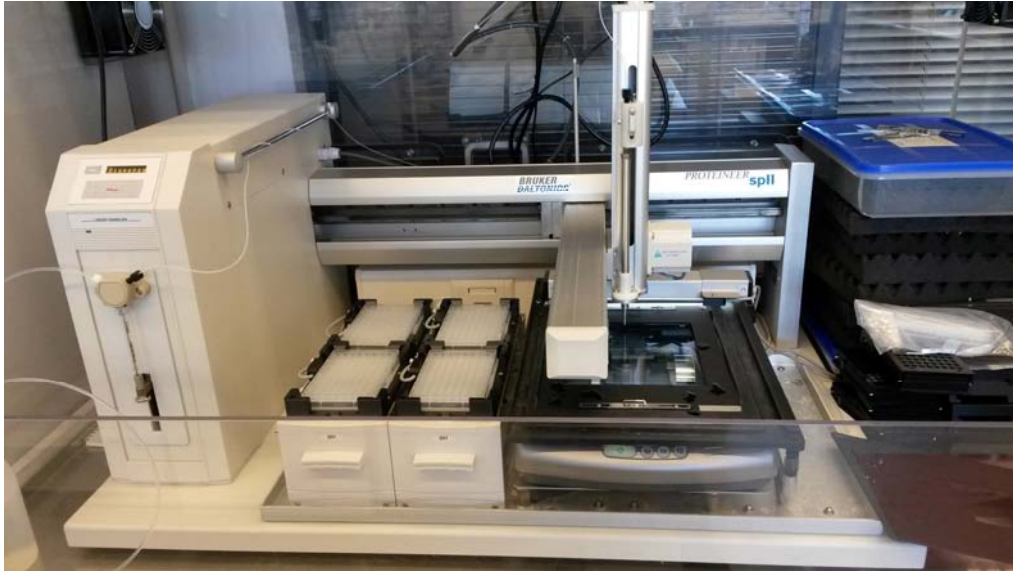
Mechanical excision was performed in the gels from samples that had been assigned for full proteomic map (Table II.i); in these gels, protein spots of interest were considered all spots on each gel. Manual excision was performed in all other gels from samples collected on D0 and subsequently; in gels of blood or milk samples collected on D0 or after challenge, as protein spots of interest were considered those that had been differentially expressed compared to gels of samples collected after challenge or on D0, respectively.

For mechanical excision, the Proteiner SPII (Bruker, Bremen, Germany) equipment was used, which excises selected protein spots and directly distributes them into the wells of a microtitre plate (Figure II.6). For manual excision, a 3 mL syringe filled with distilled water and loaded with a 200 µL plastic tip was used. The lower 5 mm of the tip had been cut off, to allow for a greater diameter; when the tip was punched into and through the gel, the protein spot for collection was retained into the plastic tip and immediately ejected into a well of a microtitre plate (Figure II.7.).

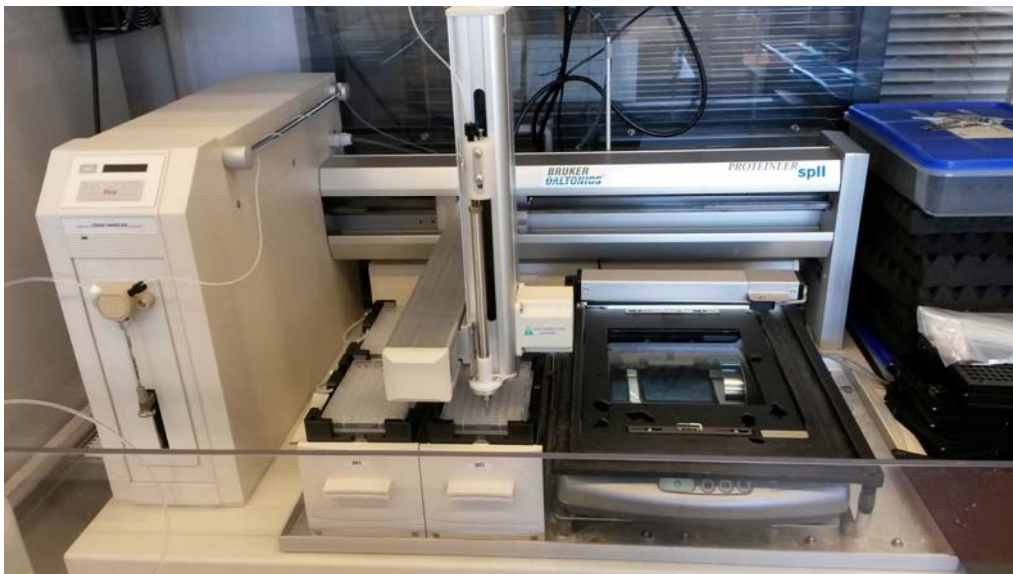


**Figure II.6.** Mechanical excision of selected protein spots from a gel (a) and ejection into a well of a microtitre plate (b).

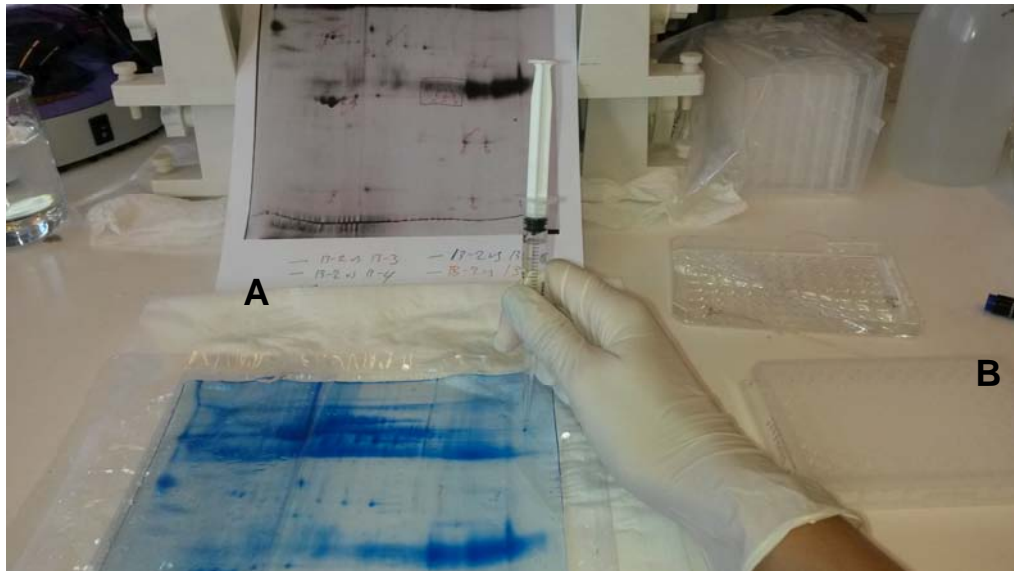
(a)



(b)



**Figure II.7.** Manual excision of protein spots of interest by means of a plastic tip, punched into and through the gel; spots had been annotated on a hard copy of the scanned gel (bold A in the background) for ejection into a well of a microtitre plate (bold B to the right of the picture).



### Tryptic digestion and peptide extraction

After collection of the spots of interest from 2-DE gels and their ejection into the wells of microtitre plates, a destain solution (140  $\mu$ L) (details in Appendix, preparation no. 11) was added in each well. Each plate was left shaking for 40 min in room temperature. The destain solution was aspirated from the wells by means of a multi-channel pipette. The procedure was repeated with distilled water used instead of the destain solution. Finally, the microtitre plate was placed for 20 min into a speed vacuum concentrator (MaxiDry Plus; Heto-Holten, Allerød, Denmark) for drying.

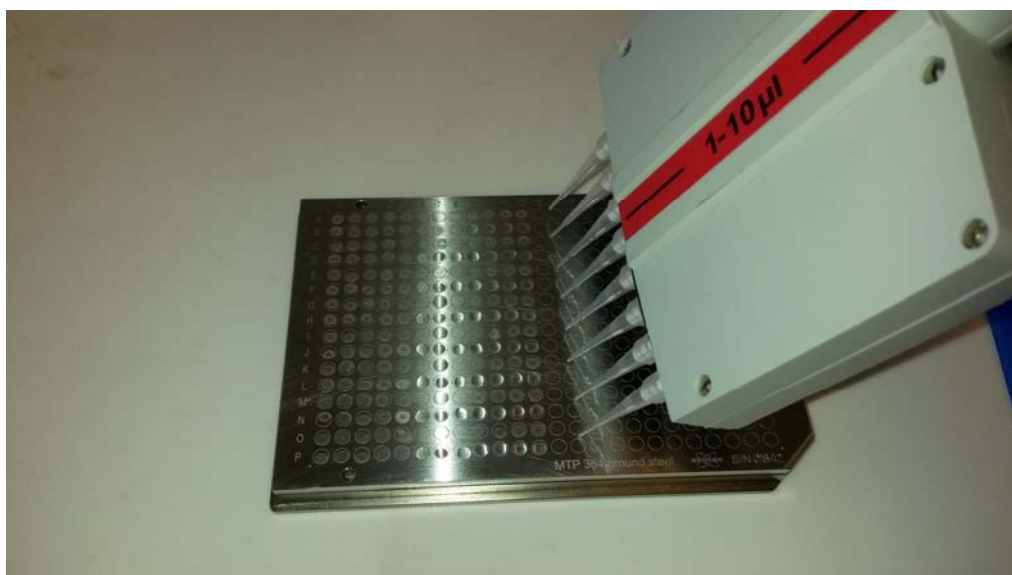
A quantity of 5  $\mu$ L of trypsin solution (details in Appendix, preparation no. 12) was added into each well of the microtitre plate, which was then covered with plastic paraffin film (Parafilm) and left at room temperature for 16 hours under dark conditions. On the following day, 10  $\mu$ L of extraction solution (details in Appendix, preparation no. 13) were added into each well, in order to extract digested peptides. The plate was covered with plastic paraffin film (Parafilm) and left shaking at room temperature for 15 min.

## Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

### *Loading of test material into the MALDI-TOF*

From each well of the microtitre plate, a quantity of 1  $\mu\text{L}$  of digested peptides was withdrawn and loaded on a MALDI target plate (Anchor Chip; Bruker) (Figure II.8.); this was followed by loading on each position of the plate 1  $\mu\text{L}$  of matrix solution (details in Appendix, preparation no. 14), which led to crystallisation of the peptide mixture. As positive controls-known peptides, a mixture of 0.5  $\mu\text{L}$  'Peptides Calibration Standards II' (Bruker) followed by matrix solution were used and placed appropriately on the plate. Finally, the target plates were loaded on the MALDI-TOF mass spectrometre (Ultraflex II; Bruker) (Figure II.9.).

**Figure II.8.** Loading of digested peptides on a MALDI target plate.



**Figure II.9.** Views of the MALDI-TOF mass spectrometre.



### *Mass spectra acquisition*

In the mass spectrometre, laser shots ( $n=400$ ) of intensity between 40% and 60% were applied for a minute period, to acquire the mass spectra by using the FlexControl software. In brief, the co-crystallised matrix-analyte was hit with the laser; irradiation of the matrix and analyte

caused a plume to form, in which proton transfer occurred between the matrix and the analyte, resulting in ionisation; the ions were accelerated by an electric field towards the TOF mass analyser, where ions of differing  $m/z$  were separated based on their flight time.

The FlexAnalysis v2.2 software (Bruker) was employed for generation of the peak list consisting of ion monoisotopic masses. Smoothing of spectra was performed using the Savitzky–Golay algorithm (width: 0.2  $m/z$ , cycle number: 1). Signal to Noise (S/N) ratio was calculated by SNAP (Sophisticated numerical annotation procedure) algorithm and a threshold S/N ratio of 2.5 was allowed. Peaks of tryptic autodigest and keratin were not taken into account. After peak picking of monoisotopic masses, the list was generated.

## **Data management and analysis**

### **Definitions**

Clinical mastitis was defined as the presence of clinically evident abnormalities in the mammary gland or the mammary secretion. Subclinical mastitis was defined as the simultaneous isolation of bacteria from milk samples and the detection of positive California Mastitis Test scores, with no clinically evident abnormalities.

### **Cytological examination of milk samples**

In the California Mastitis Test (CMT), reactions scored  $\geq 1$  were considered to be indicative of increased cellular content in milk (Fthenakis 1995).

### **Scoring of pathological findings in tissue samples**

A scoring system previously devised (Fragkou, Mavrogianni et al. 2007) was used and numerical values were assigned for the histological pathological findings in the mammary parenchyma of the experimental animals. The system is detailed in Table II.ii.

**Table II.ii.** Description of scores given for histopathological findings in the mammary parenchyma of ewes inoculated with *M. haemolytica* (Fragkou, Mavrogianni et al. 2007).

Score	Description
0	Normal
1	Presence of a few, scattered leucocytes
2	Presence of increased numbers of leucocytes clustered in the intra- and inter-alveolar area
3	Diffuse presence of leucocytes, extravasation and destruction of epithelial cells
4	Haemorrhages, destruction of alveoli and loss of the internal architecture of the parenchyma

### Total protein content in blood plasma and milk whey samples, as measured by the Bradford protein assay

The spectrophotometre provided results directly as protein content in the sample, expressed as mg mL<sup>-1</sup>. The result of each of the three dilutions tested was converted to total protein concentration in the undiluted sample; then, the median value of the three diluted samples tested was calculated and was considered as the total protein content of the original sample. Values >1.0 mg mL<sup>-1</sup> or <0.2 mg mL<sup>-1</sup> in a diluted sample were not taken into account in calculations, as per manufacturer's specifications.

### Evaluation of two-dimensional electrophoresis gels for identification of protein spots of interest

In the four gels for mechanical excision (one from blood sample, three from milk samples), all spots on each gel were considered as protein spots of interest and were annotated by using the Melanie v.4.02 software (Swiss Institute of Bioinformatics, Lausanne, Switzerland).

The remaining gels were initially evaluated visually after placing on a light source (Figure II.10). Comparisons of protein spots were made between:

- (i) gels from sequential blood samples from ewes (n=5 or 6 sampling points),
- (ii) gels from milk samples from the two mammary glands of a ewe (infected side *versus* contralateral side) on the same sampling point (n=5 or 6 pairs for each animal) and
- (iii) gels from sequential milk samples from the mammary glands of ewes (n=5 or 6 sampling points).

Subsequently, the same work was repeated by evaluating scans of the gels by using the PDQuest v.8.0 image processing software (Bio-Rad). The software offers a detailed analysis of electrophoresis gels for protein discovery; the system allows analysis of digital images of gels to



perform spot detection, spot quantification (based on absolute spot optical density) and comparisons between gels, in order to identify spots of interest. The system also provides matching, normalisation, analysis and annotation of protein spots observed on digital images of 2-DE gels. Gel spots of interest were annotated on hard copies of scans for subsequent manual excision in gels (Figure II.7.).

**Figure II.10.** 2-DE gel placed on light source in preparation for visual evaluation, for identification of protein spots of interest.



## Peptide matching and protein searches

A protein was identified by matching the results with the theoretical fragmentation patterns (*in silico* databases) by using Biotoools 3.0 software (Bruker), which employs the MASCOT search engine (Matrix Science, Boston, USA) for matching of peptides and protein searches. Peptide masses were compared with the theoretical peptide masses of all available proteins from the taxonomy 'mammalia', but excluding taxonomies '*Homo sapiens*' and 'rodents', in the UniProt Knowledge base database (UniProtKB/Swiss-Prot [release 2014 12]).

The following search parameters were applied: carbamidomethylation was considered as fixed modification, methionine oxidation was considered as variable modification and one tryptic missed cleavage was allowed. Stringent criteria were used for protein identification with a maximum allowed mass error of 25 ppm and a minimum of three matching peptides. A probability score of  $P < 0.05$  was used for affirmative protein identification. Every match produced a MOWSE

(molecular weight search) score, which was calculated as  $-10 \times \log_{10}(P)$ , where  $P$  was the calculated absolute probability. MOWSE scores over a threshold, which had been calculated automatically by the software based on the search parameters applied, led to identification and results were exported into Excel spreadsheets (Microsoft Corporation, Redmond, USA) file.

## Presentation of results of protein identification

### *Reference maps*

In the four gels, in which all protein spots had been evaluated for identification, proteins with protein score ('MASCOT' score)  $>50$  were considered for inclusion into the results. In cases of multiple identifications of the same protein in a gel (e.g., multiple spots), only the identification with the greatest score was considered. In those cases, number of spots that led to identification of same protein was recorded. In the case of the two gels from milk samples of two ewes, number of ewes in sample from which each protein had been identified, was also recorded.

Redundancy of proteins that appeared in the database under different names and accession numbers was eliminated. If over one proteins were identified under one spot, all were considered, provided that the  $>50$  protein score rule was fulfilled. For each protein, accession name, description name, MASCOT score, number of spots (only in reference protein maps), mass spectrometre coverage, molecular weight and value of isoelectric point have been incorporated in results generated by the software performing peptide matching and protein searching, as described hereabove; further, accession number, gene name, subcellular location, molecular function and biological process have been incorporated in results after appropriate searching in UniProt Knowledge base database.

Venn diagrams were designed for proteins identified or differentially expressed in the tissues examined (blood, milk from inoculated side of the udder, milk from contralateral side of the udder) by using appropriate function in Microsoft Office programs (Microsoft Corporation).

### *Differentially expressed proteins*

In gels, which were examined for differential expression of proteins, protein identification was performed as above. Differential expression of proteins after challenge (D0+12 h, D1, D2, D3, D4) was evaluated in comparison with the same protein before challenge (D0).

Proteins were considered that showed downregulation, if  
(i) they had been identified on D0, but not in any sample after challenge or



- (ii) their spot densities after challenge had been consistently and notably smaller ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0 or
- (iii) they showed a combination of i and ii.

Proteins were considered that showed new expression, if they had not been identified on D0, but had been identified in at least one sample after challenge.

Proteins were considered that showed upregulation, if their spot densities after challenge had been consistently and notably greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0.

Finally, proteins were considered that showed fluctation, if

- (i) they had been identified on D0 and intermittently after challenge or
- (ii) their spot densities had not been consistently and notably smaller or greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0.

Subcellular location, molecular function and biological process involvement of each protein were recorded from UniProt Knowledge base database.

Spot optical densities obtained from PDQuest v.8.0 for each spot of interest on each gel from sample on D0 or after challenge, were recorded. In case of multiple spots indicative of same protein, densities of all spots were taken into account. For proteins that were not differentially expressed and for proteins that fluctuated (as defined above), spot density values on D0 and after challenge were compared. Only results found to be significant during the analysis were taken into account and presented.

### *Protein clustering*

Protein classification was performed for biological process in which they were involved, by using Gene Ontology (GO) analysis (Anagnostopoulos et al. 2015).

## Statistical computations

All data were entered into Excel spreadsheets. Initially, descriptive statistics for all parametres were performed.

Comparisons were made between inoculated or contralateral sides of the animals in the proportions of (i) development of mastitis, (ii) isolations of *M. haemolytica* from milk samples and (iii) samples with increased CMT scores. Wilcoxon Signed Rank tests were performed to evaluate differences in pathology score medians in the mammary parenchyma in samples from the inoculated or the contralateral side of the udder.

For haematological parameters, the repeated measures mixed effect linear regression model was used to determine whether outcomes changed over the course of the study period. Models were adjusted for repeated measures within animals. Independent variable was day after challenge. Results obtained before challenge (D-2 and D0) were considered together.

For comparison of protein spot densities on D0 and after challenge, the repeated measures mixed effect linear regression model was used to determine whether outcomes changed over the course of the study period. Models were adjusted for repeated measures within animals. For each protein, all spots of interest obtained from all ewes on a given time-point were considered for the analysis. Independent variable was day after challenge. Data that did not fulfil the significance level as below, were reassessed and considered to be significant if median value of all spots as above, on each sampling point, was 0.5-fold change than median value on D0.

An electronic data analysis tool was employed (Vassar Stats: Website for Statistical Computation; Lowry 2012, 2015). Significance level was set at  $P \leq 0.05$ .

## **Appendix: Details of preparations used in the study**

### *Preparation no. 1*

Rehydration solution: 8 M urea (AppliChem), 2% 3-[(3-cholamidopropyl)dimethylammonio]-1 propanesulfonate (CHAPS) (Sigma-Aldrich, Saint Louis, USA), 0.4% 1,4-dithioerythritol (DTE) (Sigma-Aldrich), 0.2% IPG buffer pH 3-10 (Amersham Biosciences), few drops of bromophenol blue (Sigma-Aldrich).

### *Preparation no. 2*

IEF sample buffer: 7 M urea, 2 M thiourea (Sigma-Aldrich), 4% CHAPS, 1% DTE, 40 mM tris-HCL (pH 8.8) (Bio-Rad).

### *Preparation no. 3*

Acrylamide solution: 12% acrylamide / 1,4-bis(acryloyl)piperazine 37.5:1, 30% (Bio-Rad), 0.375 M tris-HCL (pH 8.8), 0.1% v/v SDS (Bio-Rad), 0.05% v/v tetramethylethylenediamine (AppliChem), 0.5% ammonium persulfate (Sigma-Aldrich).

*Preparation no. 4*

Equilibration stock solution: 6 M urea, 50 mM tris-HCL (pH 8.8), 30% v/v glycerol (Panreac, Castellar del Vallès, Spain), 2% v/v SDS.

*Preparation no. 5*

Equilibration working solution 1: 10 mL equilibration stock, 0.5% w/v dithioerythritol (30 mM) (Sigma-Aldrich).

*Preparation no. 6*

Equilibration working solution 2: 10 mL equilibration stock, 4.32% w/v iodoacetamide (230 mM) (Sigma-Aldrich).

*Preparation no. 7*

Agarose solution: 0.5% w/v agarose (Sigma-Aldrich), 1× Tris/glycine/SDS (TGS; Bio-Rad), a few drops of colloidal coomassie blue dye (Colloidal Blue staining kit; Thermo Fisher Scientific).

*Preparation no. 8*

SDS-PAGE electrophoresis buffer solution: 1× Tris/glycine/SDS in distilled water.

*Preparation no. 9*

Fixation solution: 50% methanol (Sigma-Aldrich), 5% phosphoric acid (Panreac).

*Preparation no. 10*

Staining solution: 20% methanol (Sigma-Aldrich), 20% Stainer A and 5% Stainer B of Colloidal Blue staining kit (colloidal coomassie blue dye) (Thermo Fisher Scientific).

*Preparation no. 11*

Destain solution: 30% acetonitrile (Fisher Scientific, Waltham, USA), 50 mM ammonium bicarbonate (Sigma-Aldrich).

*Preparation no. 12*

Trypsin solution: 100 µg lyophilised recombinant trypsin (proteomics grade; Roche Diagnostics) and 1 mL 100 mM ammonium bicarbonate added into ultra-pure water to achieve concentration of 10 mM of trypsin.

*Preparation no. 13*

Extraction solution: 50% acetonitrile (Fisher Scientific), 0.1% trifluoroacetic acid (Merck Millipore, Darmstadt, Germany).

*Preparation no. 14*

Matrix solution: 0.08%  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich), 50% acetonitrile (Fisher Scientific), 0.1% trifluoroacetic acid (Merck Millipore), plus internal standards.

Internal standards: minute quantities (Fmol) des-Arg-bradykinin (molecular weight: 904.4681 Da; Sigma-Aldrich) and adrenocorticotrophic hormone fragment 18–39 (molecular weight: 2465.1989 Da; Sigma-Aldrich).

## C. RESULTS

### Results of clinical and conventional laboratory examinations in samples from the experimental animals

#### Findings in examinations before inoculation of the experimental ewes

The mammary glands and the teats of all ewes were clinically healthy in the examination performed before challenge. No bacteria were isolated from any milk sample obtained. California Mastitis Test scores in milk samples were negative (scores 'negative' or 'trace') and observation of Giemsa-stained milk films revealed only scarce presence of macrophages therein (on average, one cell per 10 fields with the 10× objective lens). Haematological parameters were within the reference ranges (Kramer 2000, Martin and Aitken 2000, Roger 2008). Detailed results are in Tables II.iii and II.iv.

#### Clinical, bacteriological and cytological findings in milk samples after inoculation of the experimental ewes

##### *Inoculated side of the udder*

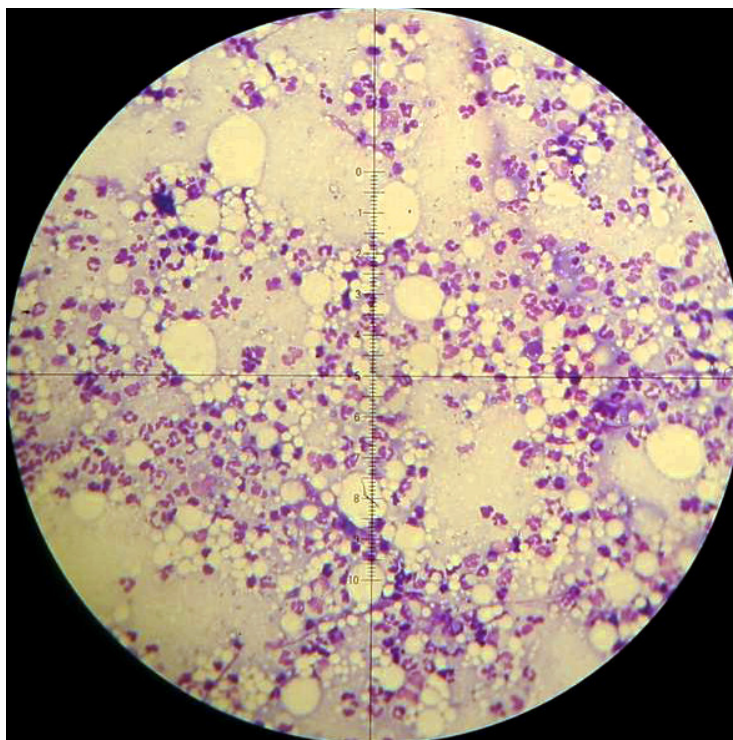
Two ewes developed (D1 to D3) signs of clinical mastitis (changes in the mammary secretion, which became serous or sero-haemorrhagic and contained flakes, and presence of increased size and temperature of the teat and the mammary parenchyma). The other three animals developed subclinical mastitis, i.e., in total, 5/5 animals developed mastitis. *M. haemolytica* was isolated in pure culture from mammary secretion samples of all ewes (in total, 12/22 isolations), starting 12 h post-inoculation and until D4. The CMT increased (>'1') in all ewes (20/22 positive samples). Leucocytes were seen in Giemsa-stained secretion films; their great majority (≥90%) consisted of neutrophils (Figure II.11), with a few macrophages and lymphocytes also present. Detailed results are in Table II.iii.

**Table II.iii.** Clinical findings, sequential isolation of *M. haemolytica* and results of California Mastitis Test in the udder of ewes with one teat inoculated with the organism.

	D-2	D0	D0+12 h	D1	D2	D3	D4
Clinical findings							
Presence	0/5	0/5	1/5	2/5	2/5	1/5	0/2
Isolation of <i>M. haemolytica</i>							
Milk	0/5	0/5	1/5	5/5	3/5	2/5	1/2
California Mastitis Test results							
Positive scores	0/5	0/5	5/5	5/5	5/5	4/5	1/2

D0: day of inoculation; D1, D2, D3, D4: days after inoculation  
n/m: positive results out of total animals sampled

**Figure II.11.** Giemsa-stained mammary secretion film, collected from inoculated side of the udder on D2, showing extensive presence of neutrophils.



#### *Non-inoculated side of the udder*

None of the contralateral side of the udder of the experimental ewes developed clinical or subclinical mastitis ( $P=0.011$  versus the inoculated sides). No bacteria were isolated from any sample from these sides (in total, 0/22 isolations,  $P<0.001$  versus the inoculated sides). No increased CMT scores were recorded in any milk sample from the non-inoculated sides of the

udder of the experimental ewes (in total, 0/22 samples with increased CMT score,  $P < 0.001$  versus the inoculated sides).

### Haematological findings after inoculation of the experimental ewes

On D1 and thereafter, there was marked decrease of haematocrit values ( $0.024 < P < 0.076$ , compared to values before challenge, depending on sampling point) and of haemoglobin concentration ( $0.001 < P < 0.017$ , compared to values before challenge, depending on sampling point), although results remained within the reference range. There was also a significant decrease of erythrocyte numbers followed by sharp increase ( $0.012 < P < 0.027$ , compared to values before challenge, depending on sampling point), with numbers recorded on D1 and D2 being below reference values. No significant changes were evident in mean corpuscular volume ( $P > 0.29$ ) and in mean corpuscular haemoglobin concentration ( $P > 0.075$ ), although in the latter parameter values below the reference range were noted.

Immediately after challenge, there was a sharp, significant increase in total leucocyte counts, which started already 12 hours after challenge and lasted up to D3 ( $0.005 < P < 0.059$ , compared to values before challenge, depending on sampling point). Increase in leucocyte counts was characterised primarily by increased counts of neutrophil and immature neutrophil counts (>2-fold increases) ( $0.001 < P < 0.019$  and  $0.008 < P < 0.25$ , respectively, compared to values before challenge, depending on sampling point). For all above cells, numbers after challenge were well over the reference range. For other leucocyte types, only lymphocyte numbers increased marginally over the reference range, which was also significant ( $P = 0.03$ ). For other cell types changes after challenge were not significant and values remained within the respective reference ranges ( $P > 0.75$ ).

Detailed results are in Table II.iv and reference values for haematological parameters in sheep are in Table II.v.

**Table II.iv.** Haematological findings (median values) in ewes with one teat inoculated with *M. haemolytica*.

Parametre	D-2 & D0	D0+12 h	D1	D2	D3	D4
Haematocrit (%)	40.7	40.7	38.5	38.4	39.0	38.3
Erythrocytes ( $\times 10^6$ cells $\mu\text{L}^{-1}$ )	10.05	9.93	9.11	9.31	13.82	13.33
Haemoglobin (g dL <sup>-1</sup> )	14.3	14.1	12.6	12.9	12.7	12.2
MCV (fL)	28.3	28.8	27.8	27.7	27.8	28.7
MCHC (g dL <sup>-1</sup> )	33.9	33.7	32.7	31.7	32.1	31.9
Total leucocytes (cells $\mu\text{L}^{-1}$ )	10,200	15,450	21,150	25,500	21,650	18,750
Neutrophils (cells $\mu\text{L}^{-1}$ )	4,284	8,253	11,175	14,823	13,230	11,387
Neutrophils (% leucocytes)	43	63	50	61	54	51
Band neutrophils (cells $\mu\text{L}^{-1}$ )	93	90	447	255	326	0
Band neutrophils (% leucocytes)	1	1	2	1	1	0
Lymphocytes (cells $\mu\text{L}^{-1}$ )	5,712	4,454	9,164	8,415	6,852	6,385
Lymphocytes (% leucocytes)	55	34	44	33	41	43
Monocytes (cells $\mu\text{L}^{-1}$ )	102	270	635	923	979	796
Monocytes (% leucocytes)	1	3	3	3	3	5
Eosinophils (cells $\mu\text{L}^{-1}$ )	0	131	447	393	384	183
Eosinophils (% leucocytes)	0	1	2	3	2	2
Basophils (cells $\mu\text{L}^{-1}$ )	0	0	0	0	0	0
Basophils (% leucocytes)	0	0	0	0	0	0
Thrombocytes (cells $\mu\text{L}^{-1}$ )	475,000	683,000	504,000	653,000	643,000	635,000

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.

MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration.

Values marked with red are outside the proposed respective reference range (Table II.v).



**Table II.v.** Reference ranges for haematological findings in sheep.

Parametre	Reference range
Haematocrit (%)	25.0-45.0
Erythrocytes ( $\times 10^6$ cells $\mu\text{L}^{-1}$ )	10.0-15.0
Haemoglobin (g $\text{dL}^{-1}$ )	9.0-15.0
MCV (fL)	25.0-30.0
MCHC (g $\text{dL}^{-1}$ )	33.0-36.0
Total leucocytes (cells $\mu\text{L}^{-1}$ )	4,000-12,000
Neutrophils (cells $\mu\text{L}^{-1}$ )	600-6,000
Neutrophils (% leucocytes)	11.0-47.0
Lymphocytes (cells $\mu\text{L}^{-1}$ )	1,500-9,000
Lymphocytes (% leucocytes)	41.0-83.0
Monocytes (% leucocytes)	0.0-13.0
Eosinophils (cells $\mu\text{L}^{-1}$ )	<1,000
Eosinophils (% leucocytes)	0.0-15.0
Basophils (% leucocytes)	0.0-3.0
Thrombocytes (cells $\mu\text{L}^{-1}$ )	180,000-750,000

MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration.

Sources for compilation of the above reference ranges of haematological parametres: Kramer (2000), Martin and Aitken (2000) and Roger (2008).

## Bacteriological findings in tissue samples after inoculation of the experimental ewes

From the inoculated side of the udder, *M. haemolytica* was isolated in pure culture in tissue samples from 2 of the 3 ewes collected on D3 and in tissue samples from one of the 2 ewes collected on D4. From the non-inoculated side of the udder, no *M. haemolytica* was isolated on any occasion.

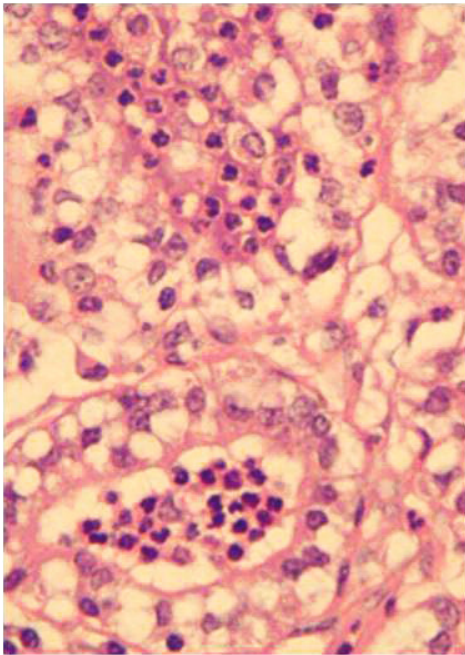
## Histopathological findings after inoculation of the experimental ewes

### *Inoculated side of the udder*

In mammary tissue samples from the inoculated side of the udder, leucocytic infiltration (neutrophils, lymphocytes) was seen histologically; furthermore, intra-alveolar live and exhausted neutrophils, extravasation and destruction of epithelial cells and alveoli were evident in some parts of the samples examined (Figure II.12). In ewes, in which clinical mastitis had developed,

conspicuous haemorrhage was also noted. All scores for histopathological findings were 3 in samples collected on D3; median score of the samples collected on D4 was 2.5.

**Figure II.12.** Histological section of mammary parenchyma, from inoculated side of the udder on D2, with marked intra-alveolar neutrophilic infiltration and destruction of mammary alveoli.



*Non-inoculated side of the udder*

No lesions were evident in any sample from the contralateral side of the udder of the experimental ewes. Median score for histopathological findings was 0 in all samples.

Scores for histopathological findings in mammary parenchyma in the inoculated side of the udder were significantly higher than those for findings in the contralateral side of the animals ( $P=0.05$ ).

## **Results of proteomics examinations in samples from the experimental animals**

### **Findings in examinations before inoculation of the experimental ewes**

#### *Findings in blood sample - 'full protein map'*

In the protein reference map that had been produced from a blood sample collected on D-2, in total, 19 proteins were identified in 155 spots on the gel. Apart from serum albumin, apolipoprotein A-I, fibrinogen beta chain and haptoglobin were also predominant (i.e., identified in  $\geq 10$  spots) proteins on the gel. Most (12/19) proteins were secreted and, in general, were involved in roles related to physiological functions in healthy animals (e.g., oxygen transport). Details of proteins identified are in Table II.vi and in Figure II.13.

**Table II.vi.** Details of proteins identified in a blood sample from a ewe, before deposition of *M. haemolytica* into one teat (identification by MALDI-TOF MS).

Accession no.	Accession name	Description name	Gene name	MASCOT score	Spots (n)	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
P12725	A1AT_SHEEP	Alpha-1-antiproteinase	N/A	95	8	32	46298	5,8	Secreted	Serine-type endopeptidase inhibitor activity	
P15497	APOA1_BOVIN	Apolipoprotein A-I	APOA1	184	16	61	30258	5,6	Secreted	Cholesterol-binding	Cholesterol metabolism, lipid metabolism, lipid transport, steroid metabolism, sterol metabolism, transport
Q32PJ2	APOA4_BOVIN	Apolipoprotein A-IV	APOA4	100	4	49	42991	5,2	Secreted	Cholesterol-binding	Lipid transport, transport
P17690	APOH_BOVIN	Beta-2-glycoprotein 1	APOH	66	2	46	39538	9,7	Secreted	Heparin binding	
Q9XT27	CERU_SHEEP	Ceruloplasmin	CP	54	4	24	120020	5,44	Secreted	Copper ion binding, ferroxidase activity	Copper transport, ion transport, transport
P02676	FIBB_BOVIN	Fibrinogen beta chain	FGB	76	11	37	53933	9,2	Secreted	Glycoprotein binding	Adaptive immunity, blood coagulation, haemostasis, immunity, innate immunity
P12799	FIBG_BOVIN	Fibrinogen gamma-B chain	FGG	60	5	27	50839	5,5	Secreted	Metal ion binding	Blood coagulation, haemostasis
Q3SX14	GELS_BOVIN	Gelsolin	GSN	112	9	26	80966	5,5	Cytoskeleton	Calcium ion binding	Cilium biogenesis/degradation
P0CH25	HBA1_CAPHI	Haemoglobin subunit alpha-1	HBA1	50	1	45	15212	9,4	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
P02075	HBB_SHEEP	Haemoglobin subunit beta	HBB	132	3	86	16120	6,9	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
P02077	HBBA_CAPHI	Haemoglobin subunit beta-A	N/A	61	2	46	16068	6,91	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
P68056	HBBC_SHEEP	Haemoglobin subunit beta-C	HBBC	51	1	40	15681	8,09	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
B6E141	HPT_CAPIB	Haptoglobin	HP	108	10	32	45411	9,1	Secreted extracellular space	Antioxidant activity	Acute phase, immunity
P81286	PLMN_SHEEP	Plasminogen (fragment)	PLG	73	8	39	38664	8,8	Secreted	Serine-type endopeptidase activity	Blood coagulation, fibrinolysis, haemostasis, tissue remodeling
P18902	RET4_BOVIN	Retinol-binding protein 4	RBP4	54	1	51	21397	5,3	Secreted	Retinal binding, retinol binding, transporter activity	Transport
Q29443	TRFE_BOVIN	Serotransferrin	TF	103	9	33	79870	6,9	Secreted	Ferric iron binding, ferric iron transmembrane transporter activity	Ion transport, iron transport, transport
P14639	ALBU_SHEEP	Serum albumin	ALB	193	52	56	71139	5,8	Extracellular space	Lipid binding, metal ion binding	Transport
P42819	SAA_SHEEP	Serum amyloid A protein	SAA1	65	1	53	12680	6,1	Secreted	Acute phase response	Acute-phase response
P12303	TTHY_SHEEP	Transthyretin	TTR	81	8	66	15875	5,5	Secreted	Thyroid hormone binding	Transport

MALDI-TOF MS: matrix assisted laser desorption/ionisation time-of-flight mass spectrometre

MS: mass spectrometre

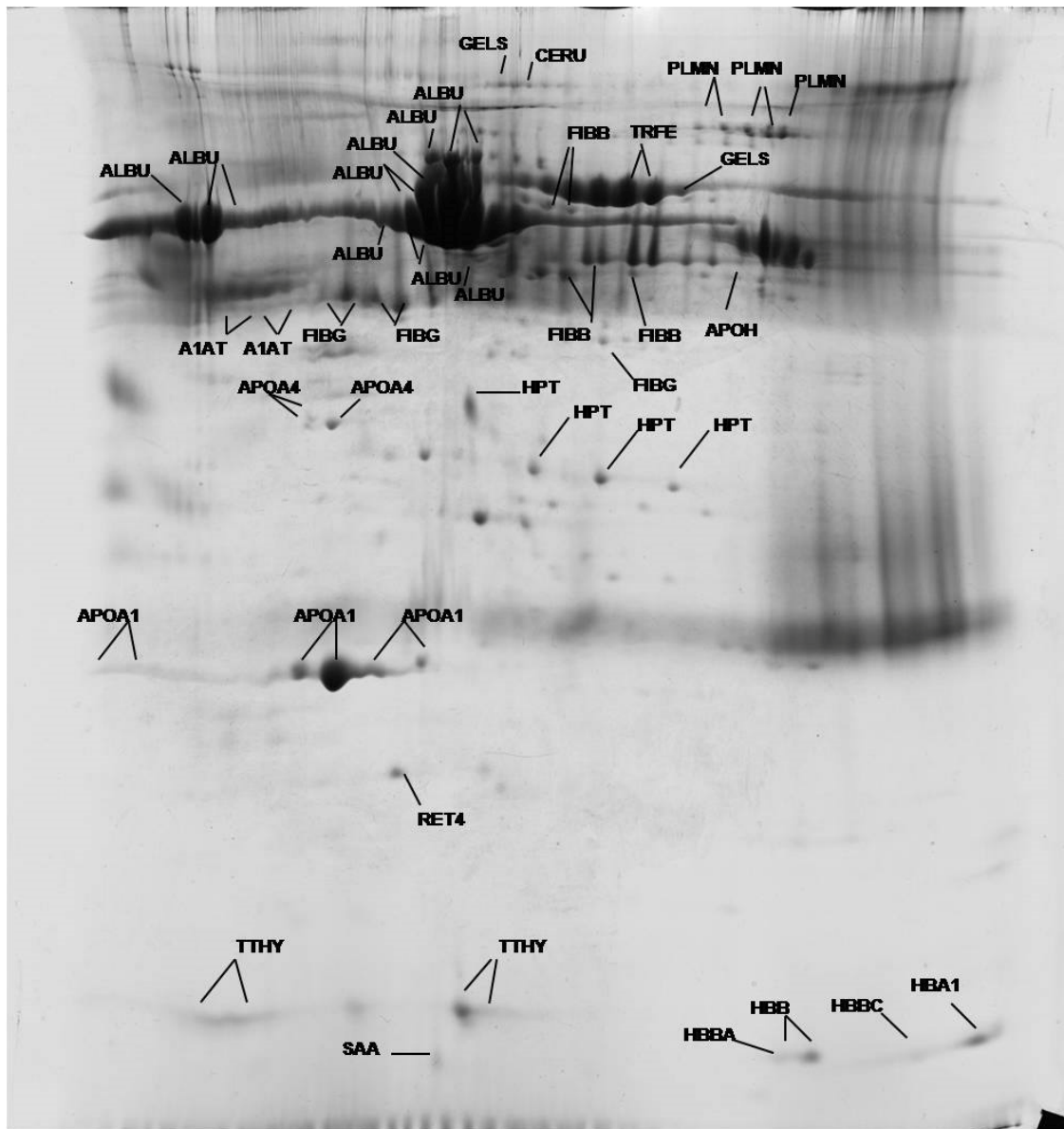
MW: molecular weight

pI: isoelectric point

PSMs: peptide spectrum matches

AAs:aminoacids

**Figure II.13.** 2-DE gel with annotation of representative protein spots, obtained from blood sample collected from a ewe, before deposition of *M. haemolytica* into one teat (protein identification by MALDI-TOF MS).



Details regarding proteins in Table II.vi.

Horizontal axis: pH 3 to 10 non-linear from left to right; vertical axis: molecular weight 10 to 100 kDa from bottom to top.

#### *Findings in milk samples - 'full protein map'*

In the protein reference maps that had been produced from two milk samples collected on D-2, in total, 40 proteins were identified in totally 280 spots on the two gels. In one sample 29 proteins were identified and in the other 17 proteins were identified, i.e., of the 40 proteins, 6 were

identified in both samples. Apart from serum albumin, alpha-lactalbumin, alpha-S2-casein, beta-lactoglobulin-1/B and lactotransferrin were also predominant (i.e., identified in  $\geq 10$  spots per gel) proteins on the gels. Most (17/40) proteins were secreted and, in general, were involved in roles related to physiological functions in healthy animals. Details of proteins identified are in Table II.vii and in Figure II.14.

**Table II.vii.** Details of proteins identified in two milk samples from two ewes, before deposition of *M. haemolytica* into one teat of each animal (identification by MALDI-TOF MS).

Accession no.	Accession name	Description name	Gene name	MASCOT score	Spots (n)	Ewe (n)	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
P60713	ACTB_SHEEP	Actin, cytoplasmic 1	ACTB	74	3	1	45	42052	5,18	Cytoskeleton	ATP-binding	Platelet aggregation, retina homeostasis, sarcomere organization
P63258	ACTG_BOVIN	Actin, cytoplasmic 2	ACTG1	75	1	1	37	42108	5,2	Cytoskeleton	ATP binding, structural constituent of cytoskeleton	
P12725	A1AT_SHEEP	Alpha-1-antiproteinase	N/A	127	8	1	34	46298	5,8	Secreted	Serine-type endopeptidase inhibitor activity	
P29701	FETUA_SHEEP	Alpha-2-HS-glycoprotein	AHSG	58	4	1	31	39511	5,1	Secreted	Cysteine-type endopeptidase inhibitor activity	Acute-phase response, negative regulation of bone mineralisation, positive regulation of phagocytosis, regulation of inflammatory response
P09462	LALBA_SHEEP	Alpha-lactalbumin	LALBA	84	24	2	35	16761	4,66	Secreted	Calcium ion binding, lactose synthase activity	Lactose biosynthesis
P04653	CASA1_SHEEP	Alpha-S1-casein	CSN1S1	63	8	2	46	24347	5,2	Secreted	Transporter activity	Cholesterol metabolism, lipid metabolism, lipid transport, steroid metabolism, sterol metabolism, transport
P04654	CASA2_SHEEP	Alpha-S2-casein	CSN1S2	71	24	2	47	26486	8,7	Secreted	Transporter activity	
P15497	APOA1_BOVIN	Apolipoprotein A-I	APOA1	148	1	1	57	30258	5,6	Secreted	Cholesterol-binding	
Q32PJ2	APOA4_BOVIN	Apolipoprotein A-IV	APOA4	66	1	1	37	42991	5,2	Secreted	Cholesterol-binding	ATP synthesis, hydrogen transport, ion transport, transport
P00829	ATPB_BOVIN	ATP synthase subunit beta, mitochondrial	ATP5B	51	1	1	25	56249	5	Mitochondria	ATP binding, proton-transporting ATPase activity, rotational mechanism	
Q6QAT4	B2MG_SHEEP	Beta-2-microglobulin	B2M	56	1	1	44	13570	6,1	Secreted	Antibacterial humoral response, immunity	
P33048	CASB_CAPHI	Beta-casein	CSN2	54	1	1	34	24906	5,1	Secreted	Transporter activity	Glycolysis
Q1KYT0	ENOB_PIG	Beta-enolase	ENO3	69	1	1	52	47443	8,93	Cytoplasm	Magnesium ion binding, phosphopyruvate hydratase activity	
P67976	LACB_SHEEP	Beta-lactoglobulin-1/B	N/A	115	79	2	59	20308	5,34	Secreted	Retinol binding	
Q5S1S4	CAH3_PIG	Carbonic anhydrase 3	CA3	62	1	1	55	29678	8,76	Cytoplasm	Carbonate dehydratase activity, nickel cation binding, zinc ion binding	One-carbon metabolic process
A2VDP1	BRE1A_BOVIN	E3 ubiquitin-protein ligase	RNF20	60	1	1	21	114272	5,6	Nucleus	Chromatin regulator, ligase	Ubl conjugation pathway
P10790	FABPH_BOVIN	Fatty acid-binding protein, heart	FABP3	57	3	1	41	14827	7,66	Cytoplasm, Mitochondrial matrix	Oleic acid binding, transporter activity	Transport
P02676	FIBB_BOVIN	Fibrinogen beta chain	FGB	106	1	1	35	53933	9,2	Secreted	Glycoprotein binding	Adaptive immunity, blood coagulation, haemostasis, immunity, innate immunity
P00883	ALDOA_RABIT	Fructose-bisphosphate aldolase A	ALDOA	50	1	1	36	39774	9,2	Sarcomere (I band, M line)	Fructose-bisphosphate aldolase activity	Glycolysis
O18751	PYGM_SHEEP	Glycogen phosphorylase, muscle form	PYGM	99	3	1	44	97702	6,7	Extracellular exosome	Glycogen phosphorylase activity, pyridoxal phosphate binding	Carbohydrate metabolism, glycogen metabolism
P01977	HBA1_TACAC	Haemoglobin subunit alpha- 1	N/A	51	1	1	46	15509	9,5	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
P02102	HBE1_CAPHI	Haemoglobin subunit epsilon-1	HBE1	52	1	1	41	16117	9,5	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
P19120	HSP7C_BOVIN	Heat shock cognate 71 kDa protein	HSPA8	86	2	1	34	71424	5,24	Cell membrane, cytoplasm, melanosome, nucleus (nucleolus)	Chaperone, repressor	mRNA processing, mRNA splicing, stress response, transcription, transcription regulation
P13943	MMP1_RABIT	Interstitial collagenase	MMP1	50	2	1	20	53877	6,3	Extracellular space (extracellular matrix)	Calcium ion binding, mettaloepeptidase activity, zinc ion binding	Collagen degradation
P02669	CASK_SHEEP	Kappa-casein	CSN3	55	5	1	35	21596	5,8	Secreted	Milk protein	Lactation, protein stabilisation
Q6EIZ0	K1C10_CANFA	Keratin, type I cytoskeletal 10	KRT10	69	6	1	25	57847	4,9	Intermediate filament	Structural molecular activity	Hydrogen peroxide
A5JUY8	PERL_BUBBU	Lactoperoxidase	LPO	53	2	1	24	81559	9,7	Secreted	Antibiotic, antimicrobial, oxidoreductase, peroxidase	
Q29477	TRFL_CAPHI	Lactotransferrin	LTF	194	15	2	49	79361	9,5	Secreted, cytoplasmic granules	Hydrolase, protease, serine protease (metal ion binding, serine-type peptidase activity)	
P00339	LDHA_PIG	L-lactate dehydrogenase A chain	LDHA	65	1	1	43	36880	9,1	Cytoplasm	Oxidoreductase (L-lactate dehydrogenase activity)	Carbohydrate metabolic process, carboxylic acid metabolic process, substantia nigra development
Q8MJV0	MYH1_HORSE	Myosin-1	MYH1	51	1	1	13	223772	5,49	Myofibril	Motor protein, muscle protein, myosin (ATP binding, motor activity)	beta-amyloid metabolic process, cellular response to cytokine stimulus, cellular response to UV-A & UV-B
Q9BE39	MYH7_BOVIN	Myosin-7	MYH7	59	9	1	14	223889	5,5	Myofibril	Motor protein, muscle protein, myosin (ATP binding, motor activity)	
P08049	NEP_RABIT	Neprilysin	MME	50	15	1	14	86212	5,2	Cell membrane (single-pass type II membrane protein)	Hydrolase, metalloprotease, protease (metalloendopeptidase activity, peptide binding, zinc ion binding)	
P81265	PIGR_BOVIN	Polymeric immunoglobulin receptor	PIGR	51	1	1	14	83695	7,7	Cell membrane (single-pass type I membrane protein)	Polymeric IgA and IgM binding at basolateral surface of epithelial cells, transporter	Glycolysis
P11979	KPYM_FELCA	Pyruvate kinase	PKM	79	2	1	40	58522	7,9	Cytoplasm, Nucleus	Kinase, transderase (ATP binding, kinase activity, magnesium ion binding, potassium ion binding, kinase activity)	

Table II.vii. (continued).

Accession no.	Accession name	Description name	Gene name	MASCOT score	Spots (n)	Ewe (n)	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
P28327	RK_BOVIN	Rhodopsin kinase	GRK1	50	1	1	23	63464	5,9	Membrane, lipid-anchor	Kinase, serine/threonine-protein kinase, transferase	Sensory transduction, vision
Q29443	TRFE_BOVIN	Serotransferrin	TF	72	1	1	24	79870	6,9	Secreted	Ferric iron binding, ferric iron transmembrane transporter activity	Ion transport, Iron transport, transport
P14639	ALBU_SHEEP	Serum albumin	ALB	292	43	2	58	71139	5,8	Extracellular space	Lipid binding, metal ion binding	Transport
P12303	TTHY_SHEEP	Transthyretin	TTR	50	2	1	55	15875	5,5	Secreted	Thyroid hormone binding	Transport
A4UMC5	TFP11_RABIT	Tuftelin-interactiing protein 11	TFIP11	52	2	1	17	96645	5,56	Cytoplasm, nucleus	DNA binding	Biomineralisation, mRNA processing, mRNA splicing
Q3MHN5	VTDB_BOVIN	Vitamin D-binding protein	GC	60	1	1	27	54904	5,2	Secreted	Vitamin D binding, vitamin transporter activity	Transport

MALDI-TOF MS: matrix assisted laser desorption/ionisation time-of-flight mass spectrometre

MS: mass spectrometre

MW: molecular weight

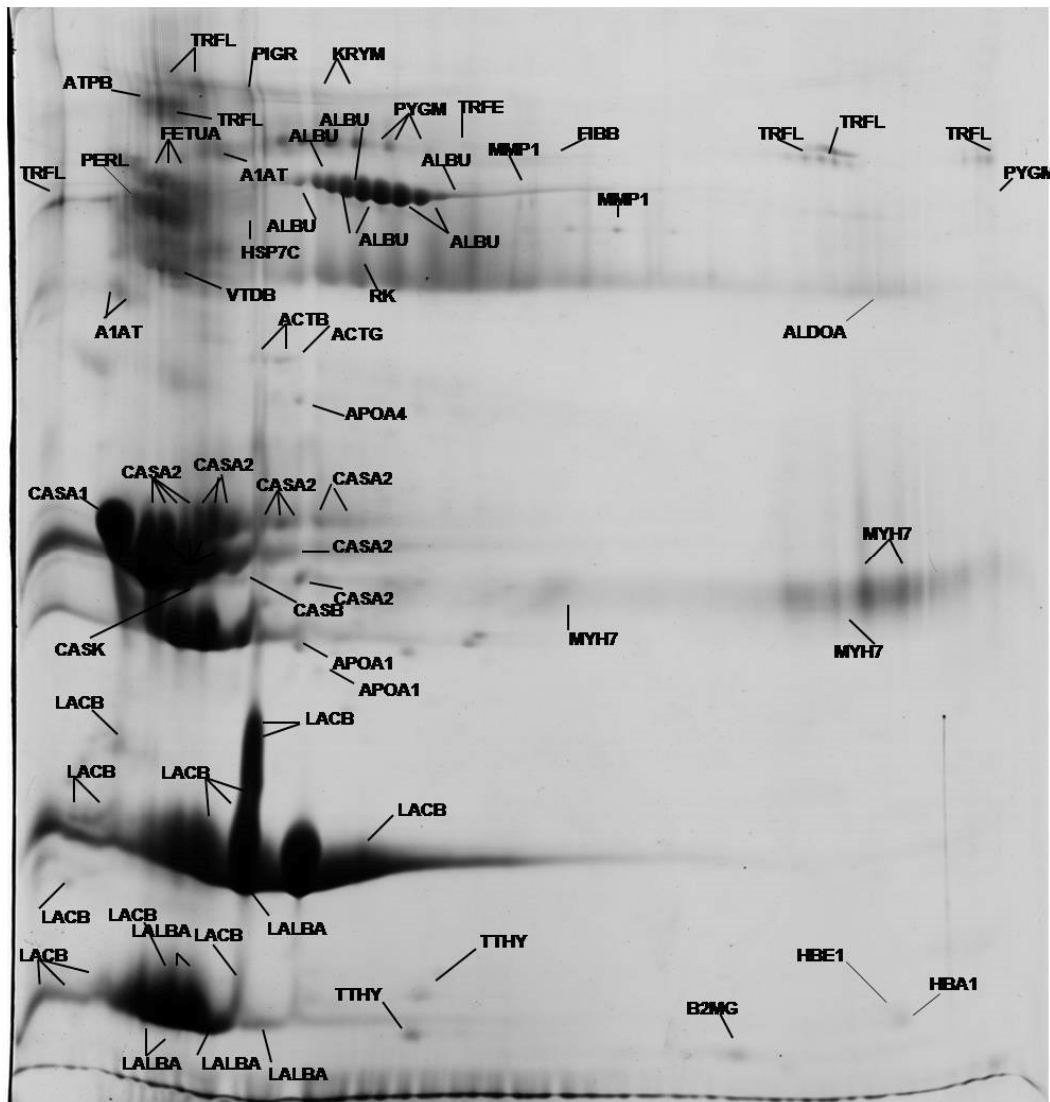
pI: isoelectric point

PSMs: peptide spectrum matches

AAs:aminoacids



**Figure II.14.** 2-DE gel with annotation of representative protein spots, obtained from milk sample from a ewe, before deposition of *M. haemolytica* into one teat (protein identification by MALDI-TOF MS).



Details regarding proteins in Table II.vii.

Horizontal axis: isoelectric point 3 to 10 (non-linear) from left to right; vertical axis: molecular weight 10 to 100 kDa (non-linear) from bottom to top.

## Proteomics findings in blood samples after inoculation of the experimental ewes

After challenge, status changes (differential expressions) were recorded in totally 33 proteins. Of these, 6 were observed with downregulation (actin, cytoplasmic 1; haemoglobin

subunit alpha-1/2; haemoglobin subunit beta-A; haemoglobin subunit beta-C; haemopexin; tuftelin-interacting protein 11) and 13 were observed with new expression (angiotensinogen; antithrombin-III; beta-2-glycoprotein 1; complement C3; complement factor B; cysteine and histidine-rich domain-containing protein 1; ETS-related transcription factor Elf-5; fibrinogen gamma-B chain; haemoglobin subunit alpha-1; haptoglobin; interleucin-4; myosin-1; peroxiredoxin-6), observed soon after challenge or with a delay, whilst 14 were observed with fluctuation (alpha-1-antiproteinase; alpha-2-macroglobulin; apolipoprotein A-I; apolipoprotein A-IV; ceruloplasmin; fibrinogen beta chain; gelsolin; haemoglobin subunit beta; plasminogen [fragment]; retinol-binding protein 4; serotransferrin; serum albumin; transthyretin; vitamin D-binding protein). Details of these proteins and their status are in Tables II.viii and II.ix and in Figure II.15.

**Table II.viii.** List of proteins observed with downregulation, new expression, upregulation or fluctuation in blood samples from five ewes, after deposition of *M. haemolytica* into one teat of each animal (protein identification by MALDI-TOF MS).

Accession name	Description name	D0	D0+ 12 h	D1	D2	D3	D4
ACTB_BOVIN	Actin, cytoplasmic 1	+	-	-	-	-	-
A1AT_SHEEP	Alpha-1-antiproteinase	+	+	+	+	+	+
A2MG_BOVIN	Alpha-2-macroglobulin	+	+	+	+	+	-
ANGT_SHEEP	Angiotensinogen	-	+	+	+	-	-
ANT3_SHEEP	Antithrombin-III	-	-	-	-	+	-
APOA1_BOVIN	Apolipoprotein A-I	+	+	+	+	+	+
APOA4_BOVIN	Apolipoprotein A-IV	+	+	+	+	+	-
APOH_BOVIN	Beta-2-glycoprotein 1	-	-	-	-	+	-
CERU_SHEEP	Ceruloplasmin	+	+	+	+	+	+
CO3_BOVIN	Complement C3	-	-	-	-	+	-
CFAB_BOVIN	Complement factor B	-	+	+	+	-	-
CHRD1_BOVIN	Cysteine and histidine-rich domain-containing protein 1	-	-	-	-	+	-
ELF5_BOVIN	ETS-related transcription factor Elf-5	-	-	-	-	+	-
FIBB_BOVIN	Fibrinogen beta chain	+	+	+	+	+	-
FIBG_BOVIN	Fibrinogen gamma-B chain	-	+	-	-	-	-
GELS_BOVIN	Gelsolin	+	+	+	+	+	+
HBA1_CAPHI	Haemoglobin subunit alpha-1	-	+	-	+	-	-

**Table II.viii** (continued).

Accession name	Description name	D0	D0+ 12 h	D1	D2	D3	D4
HBA_SHEEP	Haemoglobin subunit alpha-1/2	+	-	-	-	-	-
HBB_SHEEP	Haemoglobin subunit beta	+	+	-	-	+	-
HBBA_CAPHI	Haemoglobin subunit beta-A	+	-	-	-	-	-
HBBC_SHEEP	Haemoglobin subunit beta-C	+	-	-	-	-	-
HEMO_BOVIN	Haemopexin	+	-	-	-	-	-
HPT_CAPIB	Haptoglobin	-	-	-	-	+	-
IL4_CEREL	Interleucin-4	-	-	-	-	-	+
MYH1_BOVIN	Myosin-1	-	+	-	+	+	-
PRDX6_PIG	Peroxiredoxin-6	-	-	+	-	-	-
PLMN_SHEEP	Plasminogen (fragment)	+	+	+	+	+	+
RET4_BOVIN	Retinol-binding protein 4	+	+	+	+	+	-
TRFE_BOVIN	Serotransferrin	+	+	+	+	+	+
ALBU_SHEEP	Serum albumin	+	+	+	+	+	+
TTHY_SHEEP	Transthyretin	+	+	+	+	+	+
TFP11_RABIT	Tuftelin-interacting protein 11	+	-	-	-	-	-
VTDB_BOVIN	Vitamin D-binding protein	+	+	+	+	-	+

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.

+: protein identified in blood sample from at least one ewe, -: protein not identified in blood samples from any ewe.

Proteins were considered that showed downregulation, if (i) they had been identified on D0, but not in any sample after challenge or (ii) their spot densities after challenge had been consistently and notably smaller ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0 or (iii) they showed a combination of i and ii. Proteins were considered that showed new expression, if they had not been identified on D0, but had been in at least one sample after challenge. Proteins were considered that showed upregulation, if their spot densities after challenge had been consistently and notably greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0. Proteins were considered that showed fluctuation, if (i) they had been identified on D0 and intermittently after challenge or (ii) their spot densities had not been consistently and notably smaller or greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0.

**Table II.ix.** Details of proteins observed with downregulation, new expression, upregulation or fluctuation in blood samples from five ewes, after deposition of *M. haemolytica* into one teat of each animal (identification by MALDI-TOF MS).

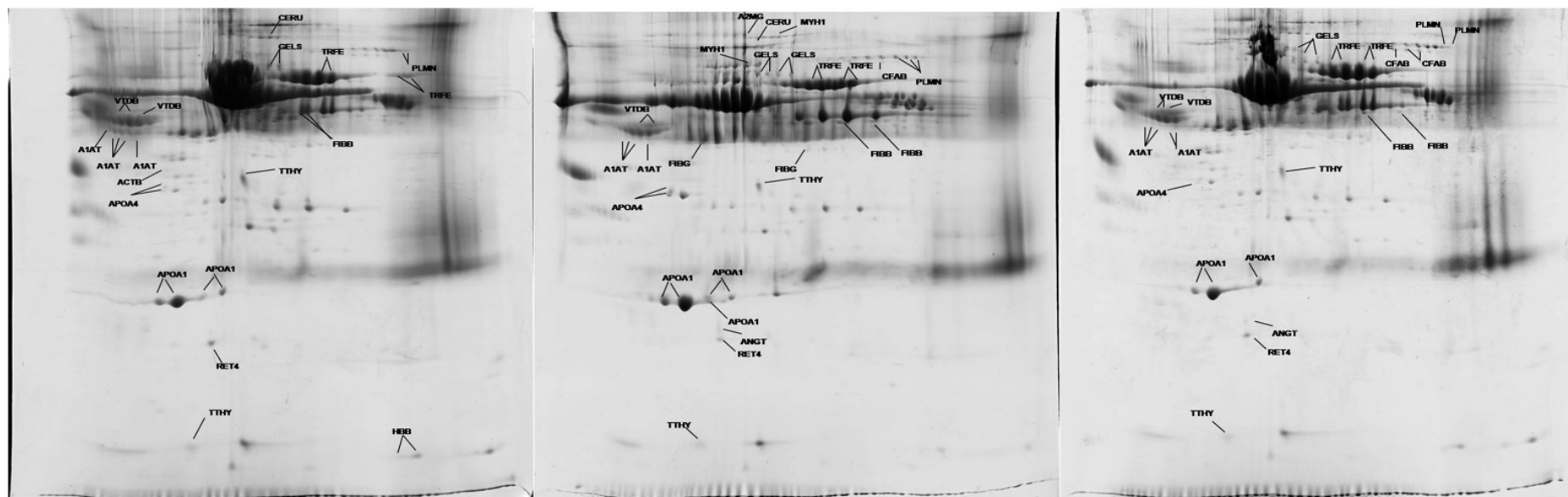
Accession no.	Accession name	Description name	Gene name	MASCOT score	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
P60713	ACTB_SHEEP	Actin, cytoplasmic 1	ACTB	96	50	42052	5,18	Cytoskeleton	ATP-binding	
P12725	A1AT_SHEEP	Alpha-1-antitrypsin	N/A	147	40	46298	5,8	Secreted	Serine-type endopeptidase inhibitor activity	
Q7SIH1	A2MG_BOVIN	Alpha-2-macroglobulin	A2M	65	19	168953	5,7	Secreted	Serine-type endopeptidase inhibitor activity	Negative regulation of complement activation, lectin pathway, stem cell differentiation
P20757	ANGT_SHEEP	Angiotensinogen	AGT	147	54	51443	6,6	Secreted	Vasoactive, vasoconstrictor	Regulation of systemic arterial blood pressure by renin-angiotensin, vasoconstriction
P32262	ANT3_SHEEP	Antithrombin-III	SERPINC1	92	36	52979	6,5	Secreted extracellular space	Protease inhibitor, serine protease inhibitor	Blood coagulation, haemostasis
P15497	APOA1_BOVIN	Apolipoprotein A-I	APOA1	139	46	30258	5,6	Secreted	Cholesterol-binding	Cholesterol metabolism, lipid metabolism, lipid transport, steroid metabolism, sterol metabolism, transport
Q32PJ2	APOA4_BOVIN	Apolipoprotein A-IV	APOA4	184	46	42991	5,2	Secreted	Cholesterol-binding	Lipid transport, Transport
P17690	APOH_BOVIN	Beta-2-glycoprotein 1	APOH	59	37	39538	9,7	Secreted	Heparin-binding	
Q9XT27	CERU_SHEEP	Ceruloplasmin	CP	122	26	120020	5,4	Secreted	Copper ion binding, ferroxidase activity	Copper transport, ion transport, transport
Q2UVX4	CO3_BOVIN	Complement C3	C3	63	15	188675	6,4	Secreted	C5L2 anaphylatoxin chemotactic receptor binding, endopeptidase inhibitor activity	Complement alternate pathway, complement pathway, fatty acid metabolism, immunity, inflammatory response, innate immunity, lipid metabolism
P81187	CFAB_BOVIN	Complement factor B	CFB	72	20	86737	8,8	Secreted	Hydrolase, protease, serine protease (serine-type endopeptidase activity)	Complement activation, alternative pathway
Q29RL2	CHRD1_BOVIN	Cysteine and histidine-rich domain-containing protein 1	CHORDC1	51	30	38144	8,69		Chaperone (ADP binding, ATP binding, zinc ion binding)	Stress response
Q58DT0	ELF5_BOVIN	ETS-related transcription factor Elf-5	ELF5	51	29	30697	5,56	Nucleus	Activator (RNA polymerase II regulatory region sequence-specific DNA binding)	Transcription, transcription regulation
P02676	FIBB_BOVIN	Fibrinogen beta chain	FGB	76	31	53933	9,2	Secreted	Glycoprotein binding	Adaptive immunity, blood coagulation, Haemostasis, immunity, innate immunity
P12799	FIBG_BOVIN	Fibrinogen gamma-B chain	FGG	63	24	50839	5,5	Secreted	Metal ion binding	Blood coagulation, haemostasis
Q3SX14	GELS_BOVIN	Gelsolin	GSN	109	29	80966	5,5	Cytoskeleton	Calcium ion binding	Cilium biogenesis/degradation
P0CH25	HBA1_CAPHI	Haemoglobin subunit alpha-1	HBA1	66	52	15212	9,4	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, Transport
P68240	HBA_SHEEP	Haemoglobin subunit alpha-1/2	N/A	66	52	15212	9,44	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, Transport
P02075	HBB_SHEEP	Haemoglobin subunit beta	HBB	114	69	16120	6,9	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, Transport
P02077	HBBA_CAPHI	Haemoglobin subunit beta-A	N/A	80	48	16068	6,91	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, Transport
P68056	HBBC_SHEEP	Haemoglobin subunit beta-C	HBBC	66	40	15681	8,09	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, Transport
Q3SZV7	HEMO_BOVIN	Haemopexin	HPX	66	16	52974	8,9	Secreted	Transport (haeme transporter activity, metal ion binding)	Transport
B6E141	HPT_CAPIB	Haptoglobin	HP	116	30	45411	9,1	Secreted	Antioxidant activity	Acute phase, Immunity
P51744	IL4_CEREL	Interleucin-4	IL4	51	42	15602	10,6	Secreted	Cytokine, growth factor (B cell activation, immune response)	B-cell activation
Q9BE40	MYH1_BOVIN	Myosin-1	MYH1	55	14	223772	5,5	Myofibril	Motor protein, muscle protein, myosin (ATP binding, motor activity)	
Q9TSX9	PRDX6_PIG	Peroxioredoxin-6	PRDX6	51	48	25078	5,66	Cytoplasm, lysosome, cytoplasmic vesicle	Antioxidant, hydrolase, oxidoreductase, peroxidase (glutathione peroxidase activity, peroxiredoxin activity, phospholipase A2 activity)	Lipid degradation, lipid metabolism
P81286	PLMN_SHEEP	Plasminogen (fragment)	PLG	85	45	38664	8,8	Secreted	Serine-type endopeptidase activity	Blood coagulation, fibrinolysis, haemostasis, tissue remodeling
P18902	RET4_BOVIN	Retinol-binding protein 4	RBP4	100	67	21397	5,3	Secreted	Retinal binding, retinol binding, transporter activity	Transport
Q29443	TRFE_BOVIN	Serotransferrin	TF	112	23	79870	6,9	Secreted	Ferric iron binding, ferric iron transmembrane transporter activity	Ion transport, iron transport, transport
P14639	ALBU_SHEEP	Serum albumin	ALB	203	58	71139	5,8	Extracellular space	Lipid binding, metal ion binding	Transport
P12303	TTHY_SHEEP	Transthyretin	TTR	107	55	15875	5,5	Secreted	Thyroid hormone binding	Transport

Table II.ix. (continued).

Accession no.	Accession name	Description name	Gene name	MASCOT score	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
A4UMC5	TFP11_RABIT	Tuftelin-interactiing protein 11	TFIP11	51	16	96645	5,6	Cytoplasm, nucleus	DNA binding	Biomineralsation, mRNA processing, mRNA splicing
Q3MHN5	VTDB_BOVIN	Vitamin D-binding protein	GC	87	31	54904	5,2	Secreted	Vitamin D binding, vitamin transporter activity	Transport

MALDI-TOF MS: matrix assisted laser desorption/ionisation time-of-flight mass spectrometre  
MS: mass spectrometre  
MW: molecular weight  
pI: isoelectric point  
PSMs: peptide spectrum matches  
AAs:aminoacids

**Figure II.15.** Sequential 2-DE gels with annotation of representative protein spots, from sequential blood samples from a ewe, before deposition of *M. haemolytica* into one teat (left), 12 h after that (centre) or 1 day after that (right) (protein identification by MALDI-TOF MS).



Details regarding proteins in Tables II.viii and II.ix.

Horizontal axis: isoelectric point 3 to 10 (non-linear) from left to right; vertical axis: molecular weight 10 to 100 kDa (non-linear) from bottom to top.

When evaluating proteins that did not show downregulation, new expression or upregulation as defined, it became evident that spot densities of some proteins decreased immediately after inoculation only for a short period (e.g., fibrinogen beta chain, transthyretin) or showed a delayed decrease (e.g., alpha-1-antiproteinase, gelsolin, serum albumin). Spot densities of other proteins increased immediately after inoculation only for a short period (e.g., alpha-2-macroglobulin, retinol-binding protein 4, vitamin D-binding protein) or showed a delayed increase (e.g., apolipoprotein A-I, apolipoprotein A-IV). Spot densities of other proteins showed a decrease or increase after inoculation followed by a subsequent increase or decrease respectively (e.g., ceruloplasmin). For some proteins, the findings were observed only in a small number of ewes (e.g., alpha-2-macroglobulin, ceruloplasmin), whilst for other results presented reflect findings in  $\geq 4$  ewes. Details are in Table II.x.

**Table II.x.** Median spot densities of proteins observed with fluctuation in gels from blood samples from five ewes, after deposition of *M. haemolytica* into one teat (protein identification by MALDI-TOF MS).

Accession name	Description name	Ewes (n)	D0	D0+ 12 h	D1	D2	D3	D4
A1AT_SHEEP	Alpha-1-antiproteinase	5	197.7	≈	≈	≈	33.6*	24.6*
A2MG_BOVIN	Alpha-2-macroglobulin	1	71.6	447.3 <sup>+</sup>	442.6 <sup>+</sup>	619.1 <sup>+</sup>	438.9 <sup>+</sup>	-
APOA1_BOVIN	Apolipoprotein A-I	3	272.9	≈	1,013.7*	1,748.2*	952.0*	460.0 <sup>+</sup>
APOA4_BOVIN	Apolipoprotein A-IV	4	198.0	≈	322.8 <sup>+</sup>	419.4*	446.5*	-
CERU_SHEEP	Ceruloplasmin	2	317.8	121.6 <sup>+</sup>	≈	606.9 <sup>+</sup>	551.2 <sup>+</sup>	1,359.9 <sup>+</sup>
FIBB_BOVIN	Fibrinogen beta chain	3	604.0	274.6 <sup>+</sup>	273.6 <sup>+</sup>	≈	≈	-
GELS_BOVIN	Gelsolin	4	302.6	≈	≈	≈	≈	64.9*
HBB_SHEEP	Haemoglobin subunit beta	3	747.1	57.1*	-	-	≈	-
PLMN_SHEEP	Plasminogen (fragment)	5	104.2	≈	≈	≈	198.4 <sup>+</sup>	≈
RET 4_BOVIN	Retinol-binding protein 4	3	314.0	515.5 <sup>+</sup>	668.6 <sup>+</sup>	956.0 <sup>+</sup>	823.2 <sup>+</sup>	-
TRFE_BOVIN	Serotransferrin	5	394.9	123.2 <sup>+</sup>	184.0 <sup>+</sup>	≈	105.3 <sup>+</sup>	124.9 <sup>+</sup>
ALBU_SHEEP	Serum albumin	5	1,022.8	≈	475.2 <sup>+</sup>	470.7 <sup>+</sup>	≈	301.0*
TTHY_SHEEP	Transthyretin	4	1,045.3	44.7*	477.6 <sup>+</sup>	452.1 <sup>+</sup>	≈	≈

**Table II.x.** (continued).

Accession name	Description name	Ewes (n)	D0	D0+12 h	D1	D2	D3	D4
VTDB_BOVIN	Vitamin D-binding protein	3	81.8	442.0 <sup>+</sup>	408.5 <sup>+</sup>	1,243.1 <sup>*</sup>	-	385.1 <sup>+</sup>

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.

≈: protein identified, but spot density not notably smaller or greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0; -: protein not identified.

\*:  $P < 0.05$ , +:  $> 0.5$ -fold change, on a given time-point compared to D0.

## Proteomics findings in milk samples after inoculation of the experimental ewes

### *Inoculated side of the udder*

#### 'Full protein map'

In the post-inoculation protein reference map that had been produced from one milk sample collected on D0+12 h, in total, 65 proteins were identified in 215 spots on the gel. Apart from serum albumin, actin cytoplasmic 1, beta-lactoglobulin-1/B and cathelicidin-1 were also predominant (i.e., identified in  $\geq 10$  spots) proteins on the gels. Most (19/65) were secreted or cytoskeleton proteins, with fewer being cytoplasm proteins (18/65); these proteins were involved in roles related to physiological functions or associated with the post-infection defence response in animals. Details of proteins identified are in Table II.xi and in Figure II.16.



**Table II.xi.** Details of proteins identified in a milk sample from a mammary gland of a ewe, 12 h after deposition of *M. haemolytica* into the ipsilateral teat (identification by MALDI-TOF MS).

Accession no.	Accession name	Description name	Gene name	MASCOT score	Spots (n)	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
Q0VCX2	GRP78_BOVIN	78 kDa glucose-regulated protein	HSPA5	62	2	19	72470	4,9	Endoplasmic reticulum lumen, melanosome, cytoplasm	ATP-binding	Maintenance of protein localisation in endoplasmic reticulum, positive regulation of cell migration
P60713	ACTB_SHEEP	Actin, cytoplasmic 1	ACTB	127	20	45	42052	5,2	Cytoskeleton	ATP-binding	Platelet aggregation, retina homeostasis, sarcomere organisation
P63258	ACTG_BOVIN	Actin, cytoplasmic 2	ACTG1	127	1	46	42108	5,2	Cytoskeleton	ATP binding, structural constituent of cytoskeleton	
A5D7D1	ACTN4_BOVIN	Alpha-actinin-4	ACTN4	95	1	22	105319	5,2	Nucleus, cytoplasm, cell junction	Calcium ion binding, Chromatin DNA binding, nucleoside binding	Protein transport, transport
Q9XSJ4	ENOA_BOVIN	Alpha-enolase	ENO1	181	7	60	47639	6,4	Cytoplasm, Cell membrane	Magnesium ion binding, phosphopyruvate hydratase activity	Glycolysis, plasminogen activation
P09462	LALBA_SHEEP	Alpha-lactalbumin	LALBA	135	3	45	16761	4,66	Secreted	Calcium ion binding, lactose synthase activity	Lactose biosynthesis
P04653	CASA1_SHEEP	Alpha-S1-casein	CSN1S1	66	6	29	24347	5,2	Secreted	Transporter activity	Cholesterol metabolism, lipid metabolism, lipid transport, steroid metabolism, sterol metabolism, transport
P04654	CASA2_SHEEP	Alpha-S2-casein	CSN1S2	112	5	38	26486	8,7	Secreted	Transporter activity	
P15497	APOA1_BOVIN	Apolipoprotein A-I	APOA1	118	2	46	30258	5,6	Secreted	Cholesterol-binding	Lipid transport, transport
Q32PJ2	APOA4_BOVIN	Apolipoprotein A-IV	APOA4	115	4	41	42991	5,2	Secreted	Cholesterol-binding	Immunity
Q6QAT4	B2MG_SHEEP	Beta-2-microglobulin	B2M	50	2	35	13570	6,1	Secreted	Antibacterial humoral response, immunity	Glycolysis
Q3ZC09	ENOB_BOVIN	Beta-enolase	ENO3	61	1	29	47409	8,63	Cytoplasm	Magnesium ion binding, phosphopyruvate hydratase activity	Transport
P67976	LACB_SHEEP	Beta-lactoglobulin-1/B	N/A	137	20	85	20308	5,34	Secreted	Retinol binding	Actin filament bundle assembly, insulin receptor signaling pathway, plasma membrane organisation, positive regulation of actin cytoskeleton reorganisation,
E1BFE9	BI2L2_BOVIN	Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2L2	51	1	13	59153	10,1	Cell membrane (peripheral membrane protein), cell junction, cytoplasmic vesicle membrane	Cytoskeletal adaptor activity, phospholipid binding	Defense response to bacteria
P54230	CTHL1_SHEEP	Cathelicidin-1	CATHL1A, CATHL1B	136	13	55	18036	9,3	Secreted	Antibiotic, antimicrobial	Defense response to bacteria
P79362	CTHL2_SHEEP	Cathelicidin-2	CATHL2	76	2	34	20057	10,89	Secreted	Antibiotic, antimicrobial	Cell death, cytotoxicity, defense response to Gram-negative bacteria, defense response to Gram-positive bacteria
P49929	SC52_SHEEP	Cathelin-related peptide SC5	N/A	50	1	25	17959	10,1	Secreted	Antibiotic, antimicrobial	Actin filament depolymerisation, cytoskeleton organisation, regulation of cell morphogenesis
Q6B7M7	COF1_SHEEP	Cofilin-1	CFL1	83	3	51	18792	9,13	Nucleus matrix, cytoskeleton, peripheral membrane protein, cytoplasmic side	Binds to F-actin and exhibits pH-sensitive F-actin depolymerizing activity	Cell shape
P31976	EZR1_BOVIN	Ezrin	EZR	50	1	12	68832	6	Peripheral membrane protein, cytoplasmic side, cytoskeleton	Actin filament binding, cell adhesion molecule binding, poly(A) RNA binding	Barbed-end actin filament capping
A4FUA8	CAZA1_BOVIN	F-actin-capping protein subunit alpha-1	CAPZA1	98	1	48	33082	5,5	Cytoskeleton	Actin capping	Adaptive immunity, blood coagulation, haemostasis, immunity, innate immunity
P02676	FIBB_BOVIN	Fibrinogen beta chain	FGB	60	5	24	53933	9,2	Secreted	Glycoprotein binding	Metabolic process
Q9TTY8	GSTP1_CAPHI	Glutathione S-transferase P	GSTP1	82	2	47	23843	8,8	Cytoplasm, mitochondrion, nucleus	Transferase (glutathione transferase activity)	Apoptosis, glycolysis, translation regulation
Q28554	G3P_SHEEP	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	GAPDH	72	4	30	36073	9,3	Cytosol, nucleus, cytoskeleton	Glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity, microtubule binding, NAD binding, NADP binding	
P02075	HBB_SHEEP	Haemoglobin subunit beta	HBB	54	2	39	16120	6,9	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
B6E141	HPT_CAPIB	Haptoglobin	HP	53	1	19	45411	9,1	Secreted	Antioxidant activity	Acute phase, immunity
P19120	HSP7C_BOVIN	Heat shock cognate 71 kDa protein	HSPA8	55	1	23	71082	5,24	Cell membrane, cytoplasm, melanosome, nucleus (nucleolus)	Chaperone, repressor	mRNA processing, mRNA splicing, stress response, transcription, transcription regulation
Q3T149	HSPB1_BOVIN	Heat shock protein beta-1	HSPB1	97	2	44	22436	6	Cytoplasm, nucleus, cytoskeleton	Chaperone	Stress response
Q76LV2	HS90A_BOVIN	Heat shock protein HSP 90- $\alpha$	HSP90AA1	94	1	27	85077	4,78	Cytoplasm, melanosome, cell membrane	Chaperone	Stress response
Q9GKX8	HS90B_HORSE	Heat shock protein HSP 90- $\beta$	HSP90AB1	77	1	26	83527	4,82	Cytoplasm, melanosome	Chaperone	Stress response
P13943	MMP1_RABIT	Interstitial collagenase	MMP1	52	1	17	53877	6,3	Extracellular space (extracellular matrix)	Calcium ion binding, metalloendopeptidase activity, zinc ion binding	Collagen degradation
Q6XUZ5	IDHC_SHEEP	Isocitrate dehydrogenase (NADP) cytoplasmic	IDH1	54	1	19	47153	6,4	Cytoplasm	Oxidoreductase [isocitrate dehydrogenase (NADP+) activity, magnesium ion binding, NAD binding]	Glyoxylate bypass, tricarboxylic acid cycle
P02669	CASK_SHEEP	Kappa-casein	CSN3	68	3	35	21596	5,8	Secreted	Milk protein	Lactation, protein stabilisation
Q6EIZ0	K1C10_CANFA	Keratin, type I cytoskeletal 10	KRT10	78	3	23	57847	4,9	Intermediate filament	Structural molecular activity	

Table II.xi. (continued)

Accession no.	Accession name	Description name	Gene name	MASCOT score	Spots (n)	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
A5JUY8	PERL_BUBBU	Lactoperoxidase	LPO	52	1	18	81559	9,7	Secreted	Antibiotic, antimicrobial, oxidoreductase, peroxidase	Hydrogen peroxide
Q29477	TRFL_CAPHI	Lactotransferrin	LTF	120	5	31	79361	9,5	Secreted, cytoplasmic granules	Hydrolase, protease, serine protease (metal ion binding, serine-type peptidase activity)	Immunity, ion transport, iron transport, osteogenesis, transport
Q50KA9	NDKA_CANFA	Nucleoside diphosphate kinase A	NME1	53	1	31	17283	5,7	Cytoplasm, nucleus	Kinase, transferase	Differentiation, endocytosis, neurogenesis, nucleotide metabolism
Q3T0Q4	NDKB_BOVIN	Nucleoside diphosphate kinase B	NME2	55	1	36	17419	9	Cytoplasm, nucleus, cell projection	Kinase, transferase	Nucleotide metabolism
O77834	PRDX6_BOVIN	Peroxiredoxin-6	PRDX6	85	2	31	25108	6	Cytoplasm, lysosome, cytoplasmic vesicle	Antioxidant, hydrolase, oxidoreductase, peroxidase	Lipid degradation, lipid metabolism
P13696	PEBP1_BOVIN	Phosphatidylethanolamine-binding protein 1	PEBP1	52	1	39	21087	7,7	Cytoplasm	Protease inhibitor, serine protease inhibitor	ATP-binding, lipid-binding, nucleotide-binding
Q3T0P6	PGK1_BOVIN	Phosphoglycerate kinase 1	PGK1	50	1	22	44973	9,5	Cytoplasm	Kinase, transferase (ATP binding, phosphoglycerate kinase activity)	Glycolysis
Q3SZ62	PGAM1_BOVIN	Phosphoglycerate mutase 1	PGAM1	113	2	54	28948	6,8	Cytosol, extracellular exosome, membrane	Hydrolase, isomerase	Glycolysis
Q3SZJ9	PMM2_BOVIN	Phosphomannomutase 2	PMM2	52	1	25	28435	6	Cytoplasm	Phosphomannomutase activity	GDP-mannose biosynthetic process
P05307	PDIA1_BOVIN	Protein disulfide-isomerase	P4HB	64	1	24	57629	4,7	Endoplasmic reticulum, melanosome, cell membrane	Chaperone, isomerase	Cell redox homeostasis, regulation of oxidative stress-induced intrinsic apoptotic signaling pathway, response to endoplasmic reticulum stress
P38657	PDIA3_BOVIN	Protein disulfide-isomerase A3	PDIA3	141	2	35	57293	6,2	Endoplasmic reticulum, melanosome	Protein disulfide isomerase activity	Cell redox homeostasis, regulation of oxidative stress-induced intrinsic apoptotic signaling pathway, response to endoplasmic reticulum stress
P28783	S10A9_BOVIN	Protein S100-A9	S100A9	50	1	44	17160	6,3	Secreted, cytoplasm, cytoskeleton, peripheral membrane protein	Antimicrobial, antioxidant	Apoptosis, autophagy, chemotaxis, immunity, inflammatory response, innate immunity
P80601	UK114_CAPHI	Ribonuclease	HRSP12	81	3	82	14347	7,1	Cell surface	Endonuclease activity	
Q29443	TRFE_BOVIN	Serotransferrin	TF	89	8	31	79870	6,9	Secreted	Ferric iron binding, ferric iron transmembrane transporter activity	Ion transport, iron transport, transport
P14639	ALBU_SHEEP	Serum albumin	ALB	205	34	40	71139	5,8	Extracellular space	Lipid binding, metal ion binding	Transport
P42819	SAA_SHEEP	Serum amyloid A protein	SAA1	105	3	58	12680	6,1	Secreted	Acute-phase	Acute-phase response
Q3ZBH0	TCPB_BOVIN	T-complex protein 1 subunit beta	CCT2	64	1	25	57781	6,2	Cytoplasm	Chaperone (ATP binding)	Binding of sperm to zona pellucida, chaperone-mediated protein complex assembly, protein folding
O19011	TGFB1_HORSE	Transforming growth factor beta-1	TGFB1	50	1	18	44631	9,72	Extracellular matrix	Growth factor, mitogen	Adaptive immune response, cell development, cell growth, cell migration, cellular response
Q5E956	TPIS_BOVIN	Triosephosphate isomerase	TPI1	181	1	78	26901	6,5	Cytosol, extracellular exosome, extracellular space, nucleus	Isomerase (triose-phosphate isomerase activity)	Gluconeogenesis, glycolysis, pentose shunt
Q5KR47	TPM3_BOVIN	Tropomyosin alpha-3 chain	TPM3	69	2	28	32856	4,53	Cytoskeleton	Muscle protein	Actin-binding
P81947	TBA1B_BOVIN	Tubulin alpha-1B chain	N/A	81	3	40	50804	4,81	Cytoskeleton	GTP-binding, nucleotide-binding	Cellular response to interleucin-4, microtubule cytoskeleton organization
Q2HJ86	TBA1D_BOVIN	Tubulin alpha-1D chain	TUBA1D	61	1	34	50935	4,77	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
Q32KN8	TBA3_BOVIN	Tubulin alpha-3 chain	TUBA3	60	1	33	50578	4,84	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
P81948	TBA4A_BOVIN	Tubulin alpha-4A chain	TUBA4A	69	2	36	50634	4,79	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
P02554	TBB_PIG	Tubulin beta chain	N/A	185	1	55	50285	4,64	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
Q6B856	TBB2B_BOVIN	Tubulin beta-2B chain	TUBB2B	173	1	53	50377	4,64	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process, neuron migration
Q3MHM5	TBB4B_BOVIN	Tubulin beta-4B chain	TUBB4B	153	1	45	50255	4,65	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
Q2KJD0	TBB5_BOVIN	Tubulin beta-5 chain	TUBB5	214	5	61	50095	4,6	Cytoskeleton	GTP-binding, nucleotide-binding	Spindle assembly
Q2HJ81	TBB6_BOVIN	Tubulin beta-6 chain	TUBB6	61	1	22	50324	4,6	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
A4UMC5	TFP11_RABIT	Tuftelin-interacting protein 11	TFIP11	50	1	18	96645	5,6	Cytoplasm, nucleus	DNA binding	Biominaleralisation, mRNA processing, mRNA splicing
P26234	VINC_PIG	Vinculin	VCL	79	1	19	124437	5,5	Peripheral membrane protein, cytoplasmic side, cell junction, cytoskeleton	Structural molecule activity	Cell adhesion
Q3MHN5	VTDB_BOVIN	Vitamin D-binding protein	GC	70	2	31	54904	5,2	Secreted	Vitamin D binding, vitamin transporter activity	Transport

MALDI-TOF MS: matrix assisted laser desorption/ionisation time-of-flight mass spectrometre

MS: mass spectrometre

MW: molecular weight

pI: isoelectric point

PSMs: peptide spectrum matches

AAs:aminoacids



epsilon-1; L-lactate dehydrogenase A chain; lactoperoxidase; myosin-1; neprilysin; pyruvate kinase; rhodopsin kinase), 53 were observed with new expression (78 kDa glucose-regulated protein; alpha-actinin-4; alpha-enolase; beta-enolase; brain-specific angiogenesis inhibitor 1-associated protein 2; caspase-1; cathelicidin-1; cathelicidin-2; cathelin-related peptide SC5; chitinase-3-like protein 1; cofilin-1; ezrin; F-actin-capping protein subunit alpha-1; galactose-3-O-sulfotransferase 3; glutathione S-transferase P; glyceraldehyde-3-phosphate dehydrogenase [fragment]; glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1; haemoglobin subunit beta; haptoglobin; heat shock protein beta-1; heat shock protein HSP 90-alpha; heat shock protein HSP 90-beta; isocitrate dehydrogenase [NADP] cytoplasmic; myosin-7; nucleoside diphosphate kinase A; nucleoside diphosphate kinase B; peroxiredoxin-6; peroxisomal membrane protein 11B; phakinin; phosphatidylethanolamine-binding protein 1; phosphoglycerate kinase 1; phosphoglycerate mutase 1; phosphomannomutase 2; protein disulfide-isomerase; protein disulfide-isomerase A3; protein S100-A9; ribonuclease; serum amyloid A protein; T-complex protein 1 subunit beta; transforming growth factor beta-1; triosephosphate isomerase; tropomyosin alpha-3 chain; tubulin alpha-1B chain; tubulin alpha-1D chain; tubulin alpha-3 chain; tubulin alpha-4A chain; tubulin beta chain; tubulin beta-2B chain; tubulin beta-4B chain; tubulin beta-5 chain; tubulin beta-6 chain; vinculin; vitamin D-binding protein) and 3 were observed with upregulation (actin, cytoplasmic 1; apolipoprotein A-I; kappa-casein), observed soon after challenge or with a delay, whilst 15 were observed with fluctuation (actin, cytoplasmic 2; alpha-lactalbumin; alpha-S1-casein; alpha-S2-casein; apolipoprotein A-IV; beta-2-microglobulin; fibrinogen beta chain; heat shock cognate 71 kDa protein; interstitial collagenase; lactotransferrin; polymeric immunoglobulin receptor; serotransferrin; serum albumin transthyretin; tuftelin-interacting protein 11). Details of these proteins and their status are in Table II.xii and II.xiii and in Figure II.17.

**Table II.xii.** List of proteins observed with downregulation, new expression, upregulation or fluctuation in milk samples from a mammary gland of five ewes, after deposition of *M. haemolytica* into the ipsilateral teat of each animal (protein identification by MALDI-TOF MS).

(a)

Accession name	Description name	D0	D0+ 12 h	D1	D2	D3	D4
GRP78_BOVIN	78 kDa glucose-regulated protein	-	+	-	-	-	-
ACTB_SHEEP	Actin, cytoplasmic 1	+	+	+	+	+	+
ACTG_BOVIN	Actin, cytoplasmic 2	+	+	+	+	-	+
A1AT_SHEEP	Alpha-1-antiproteinase	+	-	-	+	-	-
FETUA_SHEEP	Alpha-2-HS-glycoprotein	+	-	-	-	-	-
ACTN4_BOVIN	Alpha-actinin-4	-	+	-	-	-	-
ENOA_BOVIN	Alpha-enolase	-	+	+	+	+	-
LALBA_SHEEP	Alpha-lactalbumin	+	+	+	+	+	+
CASA1_SHEEP	Alpha-S1-casein	+	+	+	+	+	+
CASA2_SHEEP	Alpha-S2-casein	+	+	+	+	+	+
APOA1_BOVIN	Apolipoprotein A-I	+	+	+	+	+	+
APOA4_BOVIN	Apolipoprotein A-IV	+	+	+	+	+	-
ATPB_BOVIN	ATP synthase subunit beta, mitochondrial	+	-	-	-	-	-
B2MG_SHEEP	Beta-2-microglobulin	+	+	+	+	+	+
CASB_CAPHI	Beta-casein	+	-	-	-	-	-
ENOB_BOVIN	Beta-enolase	-	+	-	+	-	-
LACB_SHEEP	Beta-lactoglobulin-1/B	+	+	+	+	+	+
BI2L2_BOVIN	Brain-specific angiogenesis inhibitor 1-associated protein 2	-	+	+	-	-	-
CAH3_PIG	Carbonic anhydrase 3	+	-	-	-	-	-
CASP1_HORSE	Caspase-1	-	-	+	-	-	-
CTHL1_SHEEP	Cathelicidin-1	-	+	+	+	+	+
CTHL2_SHEEP	Cathelicidin-2	-	+	-	-	-	-
SC52_SHEEP	Cathelin-related peptide SC5	-	+	-	-	-	-
CH3L1_SHEEP	Chitinase-3-like protein 1	-	+	-	-	-	-
COF1_SHEEP	Cofilin-1	-	+	-	-	-	-
BRE1A_BOVIN	E3 ubiquitin-protein ligase BRE1A	+	-	-	-	-	-
EZRI_BOVIN	Ezrin	-	+	-	-	-	-
CAZA1_BOVIN	F-actin-capping protein subunit alpha-1	-	+	-	-	-	-

**Table II.xii.** (continued).

(a)

Accession name	Description name	D0	D0+ 12 h	D1	D2	D3	D4
FABPH_BOVIN	Fatty acid-binding protein, heart	+	-	-	-	-	-
FIBB_BOVIN	Fibrinogen beta chain	+	+	+	+	+	-
ALDOA_RABIT	Fructose-bisphosphate aldolase A	+	-	-	-	-	-
G3ST3_BOVIN	Galactose-3-O-sulfotransferase 3	-	-	-	+	-	-
GSTP1_CAPHI	Glutathione S-transferase P	-	+	-	-	-	-
G3P_SHEEP	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	-	+	-	-	-	-
PYGM_SHEEP	Glycogen phosphorylase, muscle form	+	-	-	-	-	-
C1GLT_BOVIN	Glycoprotein-N-acetylglactosamine 3-beta-galactosyltransferase 1	-	-	+	-	-	-
HBA1_TACAC	Haemoglobin subunit alpha-1	+	-	-	-	-	-
HBB_SHEEP	Haemoglobin subunit beta	-	+	-	+	+	-
HBE1_CAPHI	Haemoglobin subunit epsilon-1	+	-	-	-	-	-
HPT_CAPIB	Haptoglobin	-	+	+	-	-	-
HSP7C_BOVIN	Heat shock cognate 71 kDa protein	+	+	-	-	-	-
HSPB1_BOVIN	Heat shock protein beta-1	-	+	+	-	-	-
HS90A_BOVIN	Heat shock protein HSP 90-alpha	-	+	-	-	-	-
HS90B_HORSE	Heat shock protein HSP 90-beta	-	+	-	-	-	-
MMP1_RABIT	Interstitial collagenase	+	+	-	-	-	-
IDHC_SHEEP	Isocitrate dehydrogenase (NADP) cytoplasmic	-	+	-	-	-	-
CASK_SHEEP	Kappa-casein	+	+	+	+	+	+
PERL_BOVIN	Lactoperoxidase	+	+	+	-	-	-
TRFL_CAPHI	Lactotransferrin	+	+	+	+	+	+
LDHA_PIG	L-lactate dehydrogenase A chain	+	-	-	-	-	-
MYH1_HORSE	Myosin-1	+	-	-	-	-	-
MYH7_BOVIN	Myosin-7	-	-	+	-	-	-

**Table II.xii.** (continued).

(a)

Accession name	Description name	D0	D0+ 12 h	D1	D2	D3	D4
NEP_RABIT	Neprilysin	+	-	-	-	-	-
NDKA_CANFA	Nucleoside diphosphate kinase A	-	+	-	-	-	-
NDKB_BOVIN	Nucleoside diphosphate kinase B	-	+	-	-	-	-
PRDX6_BOVIN	Peroxiredoxin-6	-	+	-	-	-	-
PX11B_BOVIN	Peroxisomal membrane protein 11B	-	-	-	+	-	-
BFSP2_BOVIN	Phakinin	-	-	-	+	-	-
PEBP1_BOVIN	Phosphatidylethanolamine- binding protein 1	-	+	-	-	-	-
PGK1_BOVIN	Phosphoglycerate kinase 1	-	+	+	-	-	-
PGAM1_BOVIN	Phosphoglycerate mutase 1	-	+	+	-	-	-
PMM2_BOVIN	Phosphomannomutase 2	-	+	-	-	-	-
PIGR_BOVIN	Polymeric immunoglobulin receptor	+	+	-	+	+	-
PDIA1_BOVIN	Protein disulfide- isomerase	-	+	-	-	-	-
PDIA3_BOVIN	Protein disulfide- isomerase A3	-	+	-	-	-	-
S10A9_BOVIN	Protein S100-A9	-	+	-	-	-	-
KPYM_FELCA	Pyruvate kinase	+	-	-	-	-	-
RK_BOVIN	Rhodopsin kinase	+	-	-	-	-	-
UK114_CAPHI	Ribonuclease	-	+	-	-	-	-
TRFE_BOVIN	Serotransferrin	+	+	+	+	-	+
ALBU_SHEEP	Serum albumin	+	+	+	+	+	+
SAA_SHEEP	Serum amyloid A protein	-	+	+	+	+	+
TCPB_BOVIN	T-complex protein 1 subunit beta	-	+	-	-	-	-
TGFB1_HORSE	Transforming growth factor beta-1	-	+	-	-	-	-
TTHY_SHEEP	Transthyretin	+	+	+	-	-	-
TPIS_BOVIN	Triosephosphate isomerase	-	+	+	-	-	-
TPM3_BOVIN	Tropomyosin alpha-3 chain	-	+	-	-	-	-
TBA1B_BOVIN	Tubulin alpha-1B chain	-	+	-	-	-	-
TBA1D_BOVIN	Tubulin alpha-1D chain	-	+	-	-	-	-
TBA3_BOVIN	Tubulin alpha-3 chain	-	+	-	-	-	-

**Table II.xii.** (continued).

(a)

Accession name	Description name	D0	D0+ 12 h	D1	D2	D3	D4
TBA4A_BOVIN	Tubulin alpha-4A chain	-	+	-	-	-	-
TBB_PIG	Tubulin beta chain	-	+	-	-	-	-
TBB2B_BOVIN	Tubulin beta-2B chain	-	+	-	-	-	-
TBB4B_BOVIN	Tubulin beta-4B chain	-	+	-	-	-	-
TBB5_BOVIN	Tubulin beta-5 chain	-	+	-	-	-	-
TBB6_BOVIN	Tubulin beta-6 chain	-	+	-	-	-	-
TFP11_RABIT	Tuftelin-interacting protein 11	+	+	-	-	-	-
VINC_PIG	Vinculin	-	+	-	-	-	-
VTDB_BOVIN	Vitamin D-binding protein	-	+	-	-	-	-

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.

+: protein identified in milk sample from at least one ewe, -: protein not identified in milk samples from any ewe.

Proteins were considered that showed downregulation, if (i) they had been identified on D0, but not in any sample after challenge or (ii) their spot densities after challenge had been consistently and notably smaller ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0 or (iii) they showed a combination of I and ii. Proteins were considered that showed new expression, if they had not been identified on D0, but had been in at least one sample after challenge. Proteins were considered that showed upregulation, if their spot densities after challenge had been consistently and notably greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0. Proteins were considered that showed fluctuation, if (i) they had been identified on D0 and intermittently after challenge or (ii) their spot densities had not been consistently and notably smaller or greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0.

(b)

Accession name	Description name	Ewes (n)	D0	D0+ 12 h	D1	D2	D3	D4
ACTB_SHEEP	Actin, cytoplasmic 1	5	58.3	593.6 <sup>+</sup>	289.8 <sup>+</sup>	230.0 <sup>+</sup>	429.1 <sup>+</sup>	1,422.8 <sup>+</sup>
A1AT_SHEEP	Alpha-1-antiproteinase	2	329.7	-	-	8.2 <sup>+</sup>	-	-
APOA1_BOVIN	Apolipoprotein A-I	5	214.8	694.4 <sup>+</sup>	730.0 <sup>+</sup>	637.2 <sup>+</sup>	613.0 <sup>+</sup>	1,182.3 <sup>+</sup>
LACB_SHEEP	Beta-lactoglobulin-1/B	5	3,201.6	733.5 <sup>+</sup>	749.5 <sup>+</sup>	1,302.5 <sup>+</sup>	682.8 <sup>+</sup>	490.8 <sup>+</sup>
CASK_SHEEP	Kappa-casein	3	350.7	1,401.2 <sup>+</sup>	577.4 <sup>+</sup>	2,203.3 <sup>+</sup>	906.8 <sup>+</sup>	850.9 <sup>+</sup>
PERL_BOVIN	Lactoperoxidase	1	683.0	323.9 <sup>+</sup>	318.3 <sup>+</sup>	-	-	-

-: protein not identified in milk samples from any ewe.

\*:  $P < 0.05$ , +:  $> 0.5$ -fold change, on a given time-point compared to D0.



**Table II.xiii.** Details of proteins observed with downregulation, new expression, upregulation or fluctuation in milk samples from a mammary gland of five ewes, after deposition of *M. haemolytica* into the ipsilateral teat of each animal (identification by MALDI-TOF MS).

Accession no.	Accession name	Description name	Gene name	MASCOT score	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
Q0VCX2	GRP78_BOVIN	78 kDa glucose-regulated protein	HSPA5	62	19	72470	4,9	Endoplasmic reticulum lumen, melanosome, cytoplasm	ATP-binding	Maintenance of protein localisation in endoplasmic reticulum, positive regulation of cell migration
P60713	ACTB_SHEEP	Actin, cytoplasmic 1	ACTB	146	53	42052	5,18	Cytoskeleton	ATP-binding	
P63258	ACTG_BOVIN	Actin, cytoplasmic 2	ACTG1	146	53	42108	5,2	Cytoskeleton	ATP binding, structural constituent of cytoskeleton	Platelet aggregation, retina homeostasis, sarcomere organization
P12725	A1AT_SHEEP	Alpha-1-antiproteinase	N/A	92	32	46298	5,8	Secreted	Serine-type endopeptidase inhibitor activity	Acute-phase response, negative regulation of bone mineralisation, positive regulation of phagocytosis, regulation of inflammatory response
P29701	FETUA_SHEEP	Alpha-2-HS-glycoprotein	AHSG	58	31	39511	5,1	Secreted	Cysteine-type endopeptidase inhibitor activity	
A5D7D1	ACTN4_BOVIN	Alpha-actinin-4	ACTN4	95	22	105319	5,2	Nucleus, cytoplasm, cell junction	Calcium ion binding, Chromatin DNA binding, nucleoside binding	Protein transport, transport
Q9XSJ4	ENOA_BOVIN	Alpha-enolase	ENO1	181	60	47639	6,4	Cytoplasm, Cell membrane	Magnesium ion binding, phosphopyruvate hydratase activity	Glycolysis, plasminogen activation
P09462	LALBA_SHEEP	Alpha-lactalbumin	LALBA	135	45	16761	4,66	Secreted	Calcium ion binding, lactose synthase activity	Lactose biosynthesis
P04653	CASA1_SHEEP	Alpha-S1-casein	CSN1S1	66	29	24347	5,2	Secreted	Transporter activity	
P04654	CASA2_SHEEP	Alpha-S2-casein	CSN1S2	112	38	26486	8,7	Secreted	Transporter activity	
P15497	APOA1_BOVIN	Apolipoprotein A-I	APOA1	200	63	30258	5,6	Secreted	Cholesterol-binding	Cholesterol metabolism, lipid metabolism, lipid transport, steroid metabolism, sterol metabolism, transport
Q32PJ2	APOA4_BOVIN	Apolipoprotein A-IV	APOA4	125	48	42991	5,2	Secreted	Cholesterol-binding	Lipid transport, transport
P00829	ATPB_BOVIN	ATP synthase subunit beta, mitochondrial	ATP5B	51	25	56249	5	Mitochondria	ATP binding, proton-transporting ATPase activity, rotational mechanism	ATP synthesis, hydrogen ion transport, ion transport, transport
Q6QAT4	B2MG_SHEEP	Beta-2-microglobulin	B2M	52	40	13570	6,1	Secreted	Antibacterial humoral response, immunity	Immunity
P33048	CASB_CAPHI	Beta-casein	CSN2	54	34	24906	5,1	Secreted	Transporter activity	
Q3ZC09	ENOB_BOVIN	Beta-enolase	ENO3	61	29	47409	8,63	Cytoplasm	Magnesium ion binding, phosphopyruvate hydratase activity	Glycolysis
P67976	LACB_SHEEP	Beta-lactoglobulin-1/B	N/A	137	85	20308	5,34	Secreted	Retinol binding	Transport
E1BFE9	BI2L2_BOVIN	Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2L2	51	13	59153	10,1	Cell membrane (peripheral membrane protein), cell junction, cytoplasmic vesicle membrane	Cytoskeletal adaptor activity, phospholipid binding	Actin filament bundle assembly, insulin receptor signaling pathway, plasma membrane organisation, positive regulation of actin cytoskeleton reorganisation,
Q5S1S4	CAH3_PIG	Carbonic anhydrase 3	CA3	62	55	29678	8,76	Cytoplasm	Lyase (carbonate dehydratase activity, nickel cation binding, zinc ion binding)	One-carbon metabolic process
Q9TV13	CASP1_HORSE	Caspase-1	CASP1	50	27	45815	6,06	Cytoplasm	Hydrolase, protease, thiol protease (cysteine-type endopeptidase activity)	Apoptosis
P54230	CTHL1_SHEEP	Cathelicidin-1	CATHL1A, CATHL1B	136	55	18036	9,3	Secreted	Antibiotic, antimicrobial	Defense response to bacteria
P79362	CTHL2_SHEEP	Cathelicidin-2	CATHL2	76	34	20057	10,89	Secreted	Antibiotic, antimicrobial	Defense response to bacteria
P49929	SC52_SHEEP	Cathelin-related peptide SC5	N/A	50	25	17959	10,1	Secreted	Antibiotic, antimicrobial	Cell death, cytotoxicity, defense response to Gram-negative bacteria, defense response to Gram-positive bacteria
Q6TMG6	CH3L1_SHEEP	Chitinase-3-like protein 1	CHI3L1	98	38	43209	9,6	Secreted extracellular space, cytoplasm (perinuclear region), endoplasmic reticulum	Antimicrobial (carbohydrate binding, chitinase activity, chitin binding)	Apoptosis, inflammatory response
Q6B7M7	COF1_SHEEP	Cofilin-1	CFL1	83	51	18792	9,13	Nucleus matrix, cytoskeleton, peripheral membrane protein, cytoplasmic side	Binds to F-actin and exhibits pH-sensitive F-actin depolymerizing activity	Actin filament depolymerisation, cytoskeleton organisation, regulation of cell morphogenesis
A2VDP1	BRE1A_BOVIN	E3 ubiquitin-protein ligase BRE1A	RNF20	60	21	114272	5,6	Nucleus	Chromatin regulator, ligase	Ubl conjugation pathway
P31976	EZR1_BOVIN	Ezrin	EZR	50	12	68832	6	Peripheral membrane protein, cytoplasmic side, cytoskeleton	Actin filament binding, cell adhesion molecule binding, poly(A) RNA binding	Cell shape
A4FUA8	CAZA1_BOVIN	F-actin-capping protein subunit alpha-1	CAPZA1	98	48	33082	5,5	Cytoskeleton	Actin capping	Barbed-end actin filament capping
P10790	FABPH_BOVIN	Fatty acid-binding protein, heart	FABP3	57	41	14827	7,66	Cytoplasm, mitochondrial matrix	Oleic acid binding, transporter activity	Transport
P02676	FIBB_BOVIN	Fibrinogen beta chain	FGB	72	26	53933	9,2	Secreted	Glycoprotein binding	Adaptive immunity, blood coagulation, haemostasis, immunity, innate immunity
P00883	ALDOA_RABIT	Fructose-bisphosphate aldolase A	ALDOA	50	36	39774	9,2	Sarcomere (I band, M line)	Fructose-bisphosphate aldolase activity	Glycolysis
Q0VCH4	G3ST3_BOVIN	Galactose-3-O-sulfotransferase 3	GAL3ST3	50	24	49124	10,5	Single-pass type II membrane protein	Transferase (galactosylceramide sulfotransferase activity)	Glycolipid biosynthetic process
Q9TTY8	GSTP1_CAPHI	Glutathione S-transferase P	GSTP1	82	47	23843	8,8	Cytoplasm, mitochondrion, nucleus	Transferase (glutathione transferase activity)	Metabolic process

Table II.xiii. (continued).

Accession no.	Accession name	Description name	Gene name	MASCOT score	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
Q28554	G3P_SHEEP	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	GAPDH	72	30	36073	9,3	Cytosol, nucleus, cytoskeleton	Glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity, microtubule binding, NAD binding, NADP binding	Apoptosis, glycolysis, translation regulation
O18751	PYGM_SHEEP	Glycogen phosphorylase, muscle form	PYGM	99	44	97702	6,7	Extracellular exosome	Glycosyltransferase, transferase (glycogen phosphorylase activity pyridoxal phosphate binding	Glycogen catabolic process, carbohydrate metabolism, glycogen metabolism
Q0VC84	C1GLT_BOVIN	Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransfer	C1GALT1	50	22	43469	6	Single-pass type II membrane protein	Developmental protein, glycosyltransferase, transferase	Angiogenesis, differentiation
P01977	HBA1_TACAC	Haemoglobin subunit alpha- 1	N/A	51	46	15509	9,5	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
P02075	HBB_SHEEP	Haemoglobin subunit beta	HBB	54	39	16120	6,9	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
P02102	HBE1_CAPHI	Haemoglobin subunit epsilon-1	HBE1	52	41	16117	9,5	Haemoglobin complex	Oxygen transport, heme binding, iron ion binding, oxygen binding	Oxygen transport, transport
B6E141	HPT_CAPIB	Haptoglobin	HP	54	22	45411	9,1	Secreted	Antioxidant activity	Acute phase, immunity
P19120	HSP7C_BOVIN	Heat shock cognate 71 kDa protein	HSPA8	55	23	71082	5,24	Cell membrane, cytoplasm, melanosome, nucleus (nucleolus)	Chaperone, repressor	mRNA processing, mRNA splicing, stress response, transcription, transcription regulation
Q3T149	HSPB1_BOVIN	Heat shock protein beta-1	HSPB1	97	44	22436	6	Cytoplasm, nucleus, cytoskeleton	Chaperone	Stress response
Q76LV2	HS90A_BOVIN	Heat shock protein HSP 90- alpha	HSP90AA1	94	27	85077	4,78	Cytoplasm, melanosome, cell membrane	Chaperone	Stress response
Q9GKX8	HS90B_HORSE	Heat shock protein HSP 90- beta	HSP90AB1	77	26	83527	4,82	Cytoplasm, melanosome	Chaperone	Stress response
P13943	MMP1_RABIT	Interstitial collagenase	MMP1	52	17	53877	6,3	Extracellular space (extracellular matrix)	Calcium ion binding, metalloendopeptidase activity, zinc ion binding	Collagen degradation
Q6XUZ5	IDHC_SHEEP	Isocitrate dehydrogenase (NADP) cytoplasmic	IDH1	54	19	47153	6,4	Cytoplasm	Oxidoreductase [isocitrate dehydrogenase (NADP+) activity, magnesium ion binding, NAD binding]	Glyoxylate bypass, tricarboxylic acid cycle
P02669	CASK_SHEEP	Kappa-casein	CSN3	68	35	21596	5,8	Secreted	Milk protein	Lactation, protein stabilisation
A5JUY8	PERL_BUBBU	Lactoperoxidase	LPO	52	18	81559	9,7	Secreted	Antibiotic, antimicrobial, oxidoreductase, peroxidase	Hydrogen peroxide
Q29477	TRFL_CAPHI	Lactotransferrin	LTF	120	31	79361	9,5	Secreted, cytoplasmic granules	Hydrolase, protease, serine protease (metal ion binding, serine-type peptidase activity)	Immunity, ion transport, iron transport, osteogenesis, transport
P00339	LDHA_PIG	L-lactate dehydrogenase A chain	LDHA	65	43	36880	9,1	Cytoplasm	Oxidoreductase (L-lactate dehydrogenase activity)	Carbohydrate metabolic process, carboxylic acid metabolic process, substantia nigra development
Q8MJV0	MYH1_HORSE	Myosin-1	MYH1	51	13	223772	5,49	Myofibril	Motor protein, muscle protein, myosin (ATP binding, motor activity)	
Q9BE39	MYH7_BOVIN	Myosin-7	MYH7	50	18	85835	6	Myofibril	Motor protein, muscle protein, myosin (ATP binding, motor activity)	
P08049	NEP_RABIT	Neprilysin	MME	50	15	86212	5,2	Cell membrane (single-pass type II membrane protein)	Hydrolase, metalloprotease, protease (metalloendopeptidase activity, peptide binding, zinc ion binding)	Beta-amyloid metabolic process, cellular response to cytokine stimulus, cellular response to UV-A & UV-B
Q50KA9	NDKA_CANFA	Nucleoside diphosphate kinase A	NME1	53	31	17283	5,7	Cytoplasm, nucleus	Kinase, transferase	Differentiation, endocytosis, neurogenesis, nucleotide metabolism
Q3T0Q4	NDKB_BOVIN	Nucleoside diphosphate kinase B	NME2	55	36	17419	9	Cytoplasm, nucleus, cell projection	Kinase, transferase	Nucleotide metabolism
O77834	PRDX6_BOVIN	Peroxisedoxin-6	PRDX6	85	31	25108	6	Cytoplasm, lysosome, cytoplasmic vesicle	Antioxidant, hydrolase, oxidoreductase, peroxidase	Lipid degradation, lipid metabolism
Q148K5	PX11B_BOVIN	Peroxisomal membrane protein 11B	PEX11B	50	28	28773	11,34	Single-pass membrane protein	Involved in peroxisomal proliferation	Peroxisome biogenesis
Q28177	BFSP2_BOVIN	Phakinin	BFSP2	50	29	46150	5,54	Peripheral membrane protein, cytoplasmic side, cytoskeleton, cell cortex	Structural constituent of eye lens	Intermediate filament cytoskeleton organization
P13696	PEBP1_BOVIN	Phosphatidylethanolamine-binding protein 1	PEBP1	52	39	21087	7,7	Cytoplasm	Protease inhibitor, serine protease inhibitor	ATP-binding, lipid-binding, nucleotide-binding
Q3T0P6	PGK1_BOVIN	Phosphoglycerate kinase 1	PGK1	50	22	44973	9,5	Cytoplasm	Kinase, transferase (ATP binding, phosphoglycerate kinase activity)	Epithelial cell differentiation, glycolytic process
Q3SZ62	PGAM1_BOVIN	Phosphoglycerate mutase 1	PGAM1	113	54	28948	6,8	Cytosol, extracellular exosome, membrane	Hydrolase, isomerase	Glycolysis
Q3SZJ9	PMM2_BOVIN	Phosphomannomutase 2	PMM2	52	25	28435	6	Cytoplasm	Phosphomannomutase activity	GDP-mannose biosynthetic process
P81265	PIGR_BOVIN	Polymeric immunoglobulin receptor	PIGR	50	15	83695	7,7	Cell membrane (single-pass type I membrane protein)	Polymeric IgA and IgM binding at basolateral surface of epithelial cells, transporter	

Table II.xiii. (continued).

Accession no.	Accession name	Description name	Gene name	MASCOT score	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
P05307	PDIA1_BOVIN	Protein disulfide-isomerase	P4HB	64	24	57629	4,7	Endoplasmic reticulum, melanosome, cell membrane	Chaperone, isomerase	Cell redox homeostasis, regulation of oxidative stress-induced intrinsic apoptotic signaling pathway, response to endoplasmic reticulum stress
P38657	PDIA3_BOVIN	Protein disulfide-isomerase A3	PDIA3	141	35	57293	6,2	Endoplasmic reticulum, melanosome	Protein disulfide isomerase activity	Cell redox homeostasis, regulation of oxidative stress-induced intrinsic apoptotic signaling pathway, response to endoplasmic reticulum stress
P28783	S10A9_BOVIN	Protein S100-A9	S100A9	50	44	17160	6,3	Secreted, cytoplasm, cytoskeleton, peripheral membrane protein	Antimicrobial, antioxidant	Apoptosis, autophagy, chemotaxis, immunity, inflammatory response, innate immunity
P11979	KPYM_FELCA	Pyruvate kinase	PKM	79	40	58522	7,9	Cytoplasm, nucleus	Kinase, transferase (ATP binding, kinase activity, magnesium ion binding, potassium ion binding, pyruvate kinase activity)	Glycolysis
P28327	RK_BOVIN	Rhodopsin kinase	GRK1	50	23	63464	5,9	Membrane, lipid-anchor	Kinase, serine/threonine-protein kinase, transferase	Sensory transduction, vision
P80601	UK114_CAPHI	Ribonuclease	HRSP12	81	82	14347	7,1	Cell surface	Endonuclease activity	
Q29443	TRFE_BOVIN	Serotransferrin	TF	112	33	79870	6,9	Secreted	Ferric iron binding, ferric iron transmembrane transporter activity	Ion transport, iron transport, transport
P14639	ALBU_SHEEP	Serum albumin	ALB	190	51	71139	5,8	Extracellular space	Lipid binding, metal ion binding	Transport
P42819	SAA_SHEEP	Serum amyloid A protein	SAA1	105	58	12680	6,1	Secreted	Acute-phase	Acute-phase response
Q3ZBH0	TCPB_BOVIN	T-complex protein 1 subunit beta	CCT2	64	25	57781	6,2	Cytoplasm	Chaperone (ATP binding)	Binding of sperm to zona pellucida, chaperone-mediated protein complex assembly, protein folding
O19011	TGFB1_HORSE	Transforming growth factor beta-1	TGFB1	50	18	44631	9,72	Extracellular matrix	Growth factor, mitogen	Adaptive immune response, cell development, cell growth, cell migration, cellular response
P12303	TTHY_SHEEP	Transthyretin	TTR	79	63	15831	5,9	Secreted	Thyroid hormone binding	Transport
Q5E956	TPIS_BOVIN	Triosephosphate isomerase	TP11	181	78	26901	6,5	Cytosol, extracellular exosome, extracellular space, nucleus	Isomerase (triose-phosphate isomerase activity)	Gluconeogenesis, glycolysis, pentose shunt
Q5KR47	TPM3_BOVIN	Tropomyosin alpha-3 chain	TPM3	69	28	32856	4,53	Cytoskeleton	Muscle protein	Actin-binding
P81947	TBA1B_BOVIN	Tubulin alpha-1B chain	N/A	81	40	50804	4,81	Cytoskeleton	GTP-binding, nucleotide-binding	Cellular response to interleucin-4, microtubule cytoskeleton organisation
Q2HJ86	TBA1D_BOVIN	Tubulin alpha-1D chain	TUBA1D	61	34	50935	4,77	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
Q32KN8	TBA3_BOVIN	Tubulin alpha-3 chain	TUBA3	60	33	50578	4,84	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
P81948	TBA4A_BOVIN	Tubulin alpha-4A chain	TUBA4A	69	36	50634	4,79	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
P02554	TBB_PIG	Tubulin beta chain	N/A	185	55	50285	4,64	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
Q6B856	TBB2B_BOVIN	Tubulin beta-2B chain	TUBB2B	173	53	50377	4,64	Cytoskeleton	GTP-binding, nucleotide-binding	microtubule-based process, neuron migration
Q3MHM5	TBB4B_BOVIN	Tubulin beta-4B chain	TUBB4B	153	45	50255	4,65	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
Q2KJD0	TBB5_BOVIN	Tubulin beta-5 chain	TUBB5	214	61	50095	4,6	Cytoskeleton	GTP-binding, nucleotide-binding	spindle assembly
Q2HJ81	TBB6_BOVIN	Tubulin beta-6 chain	TUBB6	61	22	50324	4,6	Cytoskeleton	GTP-binding, nucleotide-binding	Microtubule-based process
A4UMC5	TFP11_RABIT	Tuftelin-interacting protein 11	TFIP11	50	18	96645	5,6	Cytoplasm, nucleus	DNA binding	Biomineralisation, mRNA processing, mRNA splicing
P26234	VINC_PIG	Vinculin	VCL	79	19	124437	5,5	Peripheral membrane protein, cytoplasmic side, cell junction, cytoskeleton	Structural molecule activity	Cell adhesion
Q3MHN5	VTDB_BOVIN	Vitamin D-binding protein	GC	70	31	54904	5,2	Secreted	Vitamin D binding, vitamin transporter activity	Transport

MALDI-TOF MS: matrix assisted laser desorption/ionisation time-of-flight mass spectrometre  
MS: mass spectrometre  
MW: molecular weight  
pI: isoelectric point  
PSMs: peptide spectrum matches  
AAs:aminoacids



When evaluating proteins that did not show downregulation, new expression or upregulation as defined, it became evident that spot densities of some proteins increased immediately after inoculation only for a short period (e.g., apolipoprotein A-IV, fibrinogen beta chain, transthyretin) or showed a delayed increase (e.g., beta-2-microglobulin). Spot densities of other proteins showed a decrease or increase after inoculation followed by a subsequent increase or decrease respectively (e.g., lactotransferrin, serotransferrin, tuftelin-interacting protein 11), whilst in other proteins no clear pattern emerged (e.g., alpha-lactalbumin, alpha-S2-casein). For some proteins, the findings were observed only in a small number of ewes (e.g., alpha-lactalbumin, transthyretin), whilst for other results presented reflect findings in  $\geq 4$  ewes. Details are in Table II.xiv.

**Table II.xiv.** Median spot densities of proteins observed with fluctuation in gels from milk samples from a mammary gland of five ewes, after deposition of *M. haemolytica* into the ipsilateral teat of each animal (protein identification by MALDI-TOF MS).

Accession name	Description name	Ewes (n)	D0	D0+ 12 h	D1	D2	D3	D4
ACTG_BOVIN	Actin, cytoplasmic 2	5	58.3	593.6*	289.8+	230.0+	-	1,422.8+
LALBA_SHEEP	Alpha-lactalbumin	2	350.7	703.1+	≈	207.1+	862.6+	1,066.9+
CASA1_SHEEP	Alpha-S1-casein	2	94.0	347.6+	180.9+	141.4+	573.4+	≈
CASA2_SHEEP	Alpha-S2-casein	4	1,082.7	≈	≈	526.0+	≈	3,923.7+
APOA4_BOVIN	Apolipoprotein A-IV	2	12.4	315.0+	67.6+	61.1+	97.3+	-
B2MG_SHEEP	Beta-2-microglobulin	2	532.8	≈	1,292.8+	1,903.3+	1,623.1+	3,007.4+
FIBB_BOVIN	Fibrinogen beta chain	3	37.0	232.4*	154.8*	108.3+	255.1*	-
HSP7C_BOVIN	Heat shock cognate 71 kDa protein	1	97.0	219.8+	-	-	-	-
MMP1_RABIT	Interstitial collagenase	2	51.5	149.2+	-	-	-	-
TRFL_CAPHI	Lactotransferrin	5	524.2	81.4+	793.7+	1035.9+	≈	≈
PIGR_BOVIN	Polymeric immunoglobulin receptor	3	319.0	≈	-	990.4+	2,582.4+	-
TRFE_BOVIN	Serotransferrin	4	366.1	655.7*	581.3+	≈	-	178.0*
ALBU_SHEEP	Serum albumin	5	482.5	1,040.5+	≈	837.6+	≈	764.8+
TTHY_SHEEP	Transthyretin	2	46.1	211.6+	245.3+	-	-	-

**Table II.xiv.** (continued).

Accession name	Description name	Ewes (n)	D0	D0+ 12 h	D1	D2	D3	D4
TFP11_RABIT	Tuftelin-interacting protein 11	2	16.9	149.9 <sup>+</sup>	-	-	-	-

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.

≈: protein identified, but spot density not notably smaller or greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0; -: protein not identified.

\*:  $P < 0.05$ , +:  $> 0.5$ -fold change, on a given time-point compared to D0.

#### *Non-inoculated side of the udder*

After challenge, status changes were recorded in totally 20 proteins. Of these, one was observed with downregulation (alpha-lactalbumin) and 8 were observed with new expression (alpha-tubulin N-acetyltransferase 1; cathelicidin-1; gelsolin; myosin-4; nectin-1; phosphoglucosyltransferase-1; serum amyloid A protein; tuftelin-interacting protein 11), observed soon after challenge or with a delay, whilst 11 were observed with fluctuation (alpha-1-antiproteinase; alpha-2-HS-glycoprotein; alpha-S2-casein; apolipoprotein A-I; apolipoprotein A-IV; beta-2-microglobulin; beta-lactoglobulin-1/B; kappa-casein; lactotransferrin; serotransferrin, serum albumin). Details of these proteins and their status are in Tables II.xv and II.xvi.

**Table II.xv.** List of proteins observed with downregulation, new expression, upregulation or fluctuation in milk samples from a mammary gland of five ewes, after deposition of *M. haemolytica* into the contralateral teat of each animal (protein identification by MALDI-TOF MS).

(a)

Accession name	Description name	D0	D0+ 12 h	D1	D2	D3	D4
A1AT_SHEEP	Alpha-1-antiproteinase	+	+	+	-	-	-
FETUA_SHEEP	Alpha-2-HS-glycoprotein	+	-	+	-	-	-
LALBA_CAPHI	Alpha-lactalbumin	+	+	+	+	+	-
CASA2_SHEEP	Alpha-S2-casein	+	+	+	+	+	+
ATAT_PIG	Alpha-tubulin N-acetyltransferase 1	-	-	+	-	-	-
APOA1_BOVIN	Apolipoprotein A-I	+	+	+	+	+	+
APOA4_BOVIN	Apolipoprotein A-IV	+	+	+	+	+	-
B2MG_SHEEP	Beta-2-microglobulin	+	+	+	+	+	+
LACB_SHEEP	Beta-lactoglobulin-1/B	+	+	+	+	+	+
CTHL1_SHEEP	Cathelicidin-1	-	-	+	+	+	-
GELS_BOVIN	Gelsolin	-	-	-	+	-	-

**Table II.xv.** (continued).

(a)

Accession name	Description name	D0	D0+ 12 h	D1	D2	D3	D4
CASK_SHEEP	Kappa-casein	+	+	+	+	+	-
TRFL_CAPHI	Lactotransferrin	+	-	-	-	+	+
MYH4_RABIT	Myosin-4	-	-	+	-	-	-
PVRL1_PIG	Nectin-1	-	-	-	+	-	-
PGM1_RABIT	Phosphoglucomutase-1	-	-	-	+	-	-
TRFE_BOVIN	Serotransferrin	+	+	+	+	+	-
ALBU_SHEEP	Serum albumin	+	+	+	+	+	+
SAA_SHEEP	Serum amyloid A protein	-	-	-	+	-	-
TFP11_RABIT	Tuftelin-interacting protein 11	-	-	+	-	-	-

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.

+: protein identified in milk sample from at least one ewe, -: protein not identified in milk samples from any ewe.

Proteins were considered that showed downregulation, if (i) they had been identified on D0, but not in any sample after challenge or (ii) their spot densities after challenge had been consistently and notably smaller ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0 or (iii) they showed a combination of I and ii. Proteins were considered that showed new expression, if they had not been identified on D0, but had been in at least one sample after challenge. Proteins were considered that showed upregulation, if their spot densities after challenge had been consistently and notably greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0. Proteins were considered that showed fluctuation, if (i) they had been identified on D0 and intermittently after challenge or (ii) their spot densities had not been consistently and notably smaller or greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0.

(b)

Accession name	Description name	Ewes (n)	D0	D0+ 12 h	D1	D2	D3	D4
LALBA_CAPHI	Alpha-lactalbumin	2	3,253.9	727.9 <sup>+</sup>	1,337.0 <sup>+</sup>	153.1 <sup>+</sup>	1,151.8 <sup>+</sup>	-

-: protein not identified in milk samples from any ewe.

\*:  $P < 0.05$ , +:  $> 0.5$ -fold change, on a given time-point compared to D0.

**Table II.xvi.** Details of proteins observed with downregulation, new expression, upregulation or fluctuation in milk samples from a mammary gland of five ewes, after deposition of *M. haemolytica* into the contralateral teat of each animal (identification by MALDI-TOF MS).

Accession no.	Accession name	Description name	Gene name	MASCOT score	MS coverage	MW (kDa)	pI value	Subcellular location	Molecular function	Biological process
P12725	A1AT_SHEEP	Alpha-1-antiproteinase	N/A	95	35	46298	5,8	Secreted	Serine-type endopeptidase inhibitor activity	Acute-phase response, negative regulation of bone mineralisation, positive regulation of phagocytosis, regulation of inflammatory response
P29701	FETUA_SHEEP	Alpha-2-HS-glycoprotein	AHSG	56	32	39511	5,1	Secreted	Cysteine-type endopeptidase inhibitor activity	
P09462	LALBA_SHEEP	Alpha-lactalbumin	LALBA	58	38	16700	4,9	Secreted	Calcium ion binding, lactose synthase activity	Lactose biosynthesis
P04654	CASA2_SHEEP	Alpha-S2-casein	CSN1S2	82	42	26543	9	Secreted	Transporter activity	Alpha-tubulin acetylation, neuron development, regulation of microtubule
Q767K7	ATAT_PIG	Alpha-tubulin N-acetyltransferase 1	ATAT1	46	27	34166	10,56	Secreted	Serine-type endopeptidase inhibitor activity	
P15497	APOA1_BOVIN	Apolipoprotein A-I	APOA1	169	58	30258	5,6	Secreted	Cholesterol-binding	Cholesterol metabolism, lipid metabolism, lipid transport, steroid metabolism, sterol metabolism, transport
Q32PJ2	APOA4_BOVIN	Apolipoprotein A-IV	APOA4	50	25	42991	5,2	Secreted	Cholesterol-binding	Lipid transport, transport
Q6QAT4	B2MG_SHEEP	Beta-2-microglobulin	B2M	50	35	13570	6,1	Secreted	Antibacterial humoral response, immunity	Immunity
P67976	LACB_SHEEP	Beta-lactoglobulin-1/B	N/A	121	86	20308	5,34	Secreted	Retinol binding	Transport
P54230	CTHL1_SHEEP	Cathelicidin-1	CATHL1A, CATHL1B	75	49	18036	9,3	Secreted	Antibiotic, antimicrobial	Defense response to bacteria
Q3SX14	GELS_BOVIN	Gelsolin	GSN	50	16	80966	5,5	Cytoskeleton	Calcium ion binding	Cilium biogenesis/degradation
P02669	CASK_SHEEP	Kappa-casein	CSN3	51	36	21596	5,76	Secreted	Milk protein	Lactation, protein stabilisation
Q29477	TRFL_CAPHI	Lactotransferrin	LTF	191	43	79361	9,5	Secreted, cytoplasmic granules	Hydrolase, protease, serine protease (metal ion binding, serine-type peptidase activity)	Immunity, ion transport, iron transport, osteogenesis, transport
Q28641	MYH4_RABIT	Myosin-4	MYH4	50	13	223841	5,5	Myofibril	Motor protein, muscle protein, myosin (ATP binding, motor activity)	
Q9GL76	PVRL1_PIG	Nectin-1	NECTIN1	50	22	57410	5,8	Cell membrane (single-pass type I membrane protein)	Receptor (cell adhesion, molecule binding, protein homodimerization, receptor activity)	Cell adhesion
P00949	PGM1_RABIT	Phosphoglucomutase-1	PGM1	50	20	61805	6,65	Cytoplasm, sarcoplasmic reticulum	Isomerase	Glycogen catabolic process, carbohydrate metabolism, glycogen metabolism
Q29443	TRFE_BOVIN	Serotransferrin	TF	94	29	79870	6,9	Secreted	Ferric iron binding, ferric iron transmembrane transporter activity	Ion transport, iron transport, transport
P14639	ALBU_SHEEP	Serum albumin	ALB	232	54	71139	5,8	Extracellular space	Lipid binding, metal ion binding	Transport
P42819	SAA_SHEEP	Serum amyloid A protein	SAA1	69	43	12680	6,1	Secreted	Acute-phase	Acute-phase response
A4UMC5	TFP11_RABIT	Tuftelin-interacting protein 11	TFIP11	50	16	96645	5,6	Cytoplasm, nucleus	DNA binding	Biom mineralisation, mRNA processing, mRNA splicing

MALDI-TOF MS: matrix assisted laser desorption/ionisation time-of-flight mass spectrometre

MS: mass spectrometre

MW: molecular weight

pI: isoelectric point

PSMs: peptide spectrum matches

AAs:aminoacids



When evaluating proteins that did not show downregulation, new expression or upregulation as defined, it became evident that spot densities of some proteins decreased immediately after inoculation only for a short period (e.g., kappa-casein, lactotransferrin) or showed a delayed decrease (e.g., beta-lactoglobulin-1/B). Spot densities of other proteins increased immediately after inoculation only for a short period (e.g., alpha-1-antiproteinase, apolipoprotein A-IV, serum albumin) or showed a delayed increase (e.g., alpha-S2-casein). Spot densities of other proteins showed a decrease or increase after inoculation followed by a subsequent increase or decrease respectively (e.g., apolipoprotein A-I), whilst in other proteins no clear pattern emerged (e.g., beta-2-microglobulin, serotransferrin). For some proteins, the findings were observed only in a small number of ewes (e.g., alpha-2-HS-glycoprotein, apolipoprotein A-IV), whilst for other results presented reflect findings in  $\geq 4$  ewes. Details are in Table II.xvii.

**Table II.xvii.** Median spot densities of proteins observed with fluctuation in gels from milk samples from a mammary gland of five ewes, after deposition of *M. haemolytica* into the contralateral teat of each animal (protein identification by MALDI-TOF MS).

Accession name	Description name	Ewes (n)	D0	D0+ 12 h	D1	D2	D3	D4
A1AT_SHEEP	Alpha-1-antiproteinase	2	271.3	521.7 <sup>+</sup>	≈	-	-	-
FETUA_SHEEP	Alpha-2-HS-glycoprotein	1	89.4	-	319.2 <sup>+</sup>	-	-	-
CASA2_SHEEP	Alpha-S2-casein	4	594.5	≈	1,320.1 <sup>*</sup>	≈	≈	≈
APOA1_BOVIN	Apolipoprotein A-I	4	1,346.0	630.9 <sup>+</sup>	186.8 <sup>+</sup>	3,126.2 <sup>+</sup>	≈	≈
APOA4_BOVIN	Apolipoprotein A-IV	2	53.2	99.0 <sup>+</sup>	131.5 <sup>+</sup>	≈	≈	-
B2MG_SHEEP	Beta-2-microglobulin	2	617.8	1,212.5 <sup>+</sup>	≈	1,757.2 <sup>+</sup>	1,148.0 <sup>+</sup>	1,151.9 <sup>+</sup>
LACB_SHEEP	Beta-lactoglobulin-1/B	5	1,774.5	≈	≈	666.2 <sup>+</sup>	610.9 <sup>+</sup>	12.5 <sup>+</sup>
CASK_SHEEP	Kappa-casein	3	1,192.7	384.5 <sup>*</sup>	321.1 <sup>*</sup>	158.4 <sup>+</sup>	≈	-
TRFL_CAPHI	Lactotransferrin	4	578.5	-	-	-	≈	1,309.5 <sup>+</sup>
TRFE_BOVIN	Serotransferrin	4	517.6	860.4 <sup>+</sup>	≈	876.9 <sup>+</sup>	≈	-
ALBU_SHEEP	Serum albumin	5	543.9	983.1 <sup>+</sup>	825.4 <sup>+</sup>	≈	≈	≈

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.

≈: protein identified, but spot density not notably smaller or greater ( $P < 0.05$  or  $> 0.5$ -fold change) than that on D0; -: protein not identified.

\*:  $P < 0.05$ , +:  $> 0.5$ -fold change, on a given time-point compared to D0.

*Inoculated side versus non-inoculated side of the udder*

After challenge, differences (= lack of differential expression of a protein in samples from the contralateral side of the udder of any ewe, in case of differential expression at either side of one ewe) in expression of proteins between inoculated and non-inoculated side of the udder were recorded in 79 proteins. Of these, 74 proteins were identified in samples from the inoculated side and 5 in samples from the non-inoculated side. List of these proteins and the side of the udder in milk samples of which they were observed with differential expression, are in Table II.xviii.

**Table II.xviii.** List of proteins observed with differential expression in milk samples of only one mammary gland of five ewes, after deposition of *M. haemolytica* into one teat of each animal (protein identification by MALDI-TOF MS).

Accession name	Description name	Inoculated side	Non-inoculated side
GRP78_BOVIN	78 kDa glucose-regulated protein	+	-
ACTB_SHEEP	Actin, cytoplasmic 1	+	-
ACTG_BOVIN	Actin, cytoplasmic 2	+	-
ACTN4_BOVIN	Alpha-actinin-4	+	-
ENOA_BOVIN	Alpha-enolase	+	-
CASA1_SHEEP	Alpha-S1-casein	+	-
ATPB_BOVIN	ATP synthase subunit beta, mitochondrial	+	-
ATAT_PIG	Alpha-tubulin N-acetyltransferase	-	+
CASB_CAPHI	Beta-casein	+	-
ENOB_BOVIN	Beta-enolase	+	-
BI2L2_BOVIN	Brain-specific angiogenesis inhibitor 1-associated protein 2	+	-
CAH3_PIG	Carbonic anhydrase 3	+	-
CASP1_HORSE	Caspase-1	+	-
CTHL2_SHEEP	Cathelicidin-2	+	-
SC52_SHEEP	Cathelin-related peptide	+	-
CH3L1_SHEEP	Chitinase-3-like protein 1	+	-
COF1_SHEEP	Cofilin-1	+	-
BRE1A_BOVIN	E3 ubiquitin-protein ligase BRE1A	+	-
EZRI_BOVIN	Ezrin	+	-
CAZA1_BOVIN	F-actin-capping protein subunit alpha-1	+	-
FABPH_BOVIN	Fatty acid-binding protein, heart	+	-
FIBB_BOVIN	Fibrinogen beta chain	+	-
ALDOA_RABIT	Fructose-bisphosphate aldolase A	+	-

**Table II.xviii.** (continued).

Accession name	Description name	Inoculated side	Non-inoculated side
G3ST3_BOVIN	Galactose-3-O-sulfotransferase 3	+	-
GELS_BOVIN	Gelsolin	-	+
GSTP1_CAPHI	Glutathione S-transferase P	+	-
G3P_SHEEP	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	+	-
PYGM_SHEEP	Glycogen phosphorylase, muscle form	+	-
C1GLT_BOVIN	Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1	+	-
HBA1_TACAC	Haemoglobin subunit alpha- 1	+	-
HBB_SHEEP	Haemoglobin subunit beta	+	-
HBE1_CAPHI	Haemoglobin subunit epsilon-1	+	-
HPT_CAPIB	Haptoglobin	+	-
HSP7C_BOVIN	Heat shock cognate 71 kDa protein	+	-
HSPB1_BOVIN	Heat shock protein beta-1	+	-
HS90A_BOVIN	Heat shock protein HSP 90-alpha	+	-
HS90B_HORSE	Heat shock protein HSP 90-beta	+	-
MMP1_RABIT	Interstitial collagenase	+	-
IDHC_SHEEP	Isocitrate dehydrogenase (NADP) cytoplasmic	+	-
PERL_BOVIN	Lactoperoxidase	+	-
LDHA_PIG	L-lactate dehydrogenase A chain	+	-
MYH1_HORSE	Myosin-1	+	-
MYH4_RABIT	Myosin-4	-	+
MYH7_BOVIN	Myosin-7	+	-
PVRL1_PIG	Nectin-1	-	+
NEP_RABIT	Neprilysin	+	-
NDKA_CANFA	Nucleoside diphosphate kinase A	+	-
NDKB_BOVIN	Nucleoside diphosphate kinase B	+	-
PRDX6_BOVIN	Peroxiredoxin-6	+	-
PX11B_BOVIN	Peroxisomal membrane protein 11B	+	-
BFSP2_BOVIN	Phakinin	+	-
PEBP1_BOVIN	Phosphatidylethanolamine-binding protein 1	+	-
PGM1_RABIT	Phosphoglucomutase-1	-	+
PGK1_BOVIN	Phosphoglycerate kinase 1	+	-
PGAM1_BOVIN	Phosphoglycerate mutase 1	+	-
PMM2_BOVIN	Phosphomannomutase 2	+	-
PIGR_BOVIN	Polymeric immunoglobulin receptor	+	-
PDIA1_BOVIN	Protein disulfide-isomerase	+	-
PDIA3_BOVIN	Protein disulfide-isomerase A3	+	-

**Table II.xviii.** (continued).

Accession name	Description name	Inoculated side	Non-inoculated side
S10A9_BOVIN	Protein S100-A9	+	-
KPYM_FELCA	Pyruvate kinase	+	-
RK_BOVIN	Rhodopsin kinase	+	-
UK114_CAPHI	Ribonuclease	+	-
TCPB_BOVIN	T-complex protein 1 subunit beta	+	-
TGFB1_HORSE	Transforming growth factor beta-1	+	-
TTHY_SHEEP	Transthyretin	+	-
TPIS_BOVIN	Triosephosphate isomerase	+	-
TPM3_BOVIN	Tropomyosin alpha-3 chain	+	-
TBA1B_BOVIN	Tubulin alpha-1B chain	+	-
TBA1D_BOVIN	Tubulin alpha-1D chain	+	-
TBA3_BOVIN	Tubulin alpha-3 chain	+	-
TBA4A_BOVIN	Tubulin alpha-4A chain	+	-
TBB_PIG	Tubulin beta chain	+	-
TBB2B_BOVIN	Tubulin beta-2B chain	+	-
TBB4B_BOVIN	Tubulin beta-4B chain	+	-
TBB5_BOVIN	Tubulin beta-5 chain	+	-
TBB6_BOVIN	Tubulin beta-6 chain	+	-
VINC_PIG	Vinculin	+	-
VTDB_BOVIN	Vitamin D-binding protein	+	-

+: protein observed with differential expression in milk sample from respective mammary gland from at least one ewe; -: protein not observed with differential expression in milk sample from respective mammary gland in any ewe.

Further, differential expression in both sides of the udder was observed in 15 proteins. List of these proteins is Table II.xix.

**Table II.xix.** List of proteins observed with differential expression in milk samples of both mammary glands of five ewes, after deposition of *M. haemolytica* into one teat of each animal (protein identification by MALDI-TOF MS).

Accession name	Description name	Inoculated side	Non-inoculated side
A1AT_SHEEP	Alpha-1-antiproteinase	+	+
FETUA_SHEEP	Alpha-2-HS-glycoprotein	+	+
LALBA_SHEEP	Alpha-lactalbumin	+	+
CASA2_SHEEP	Alpha-S2-casein	+	+

**Table II.xix.** (continued).

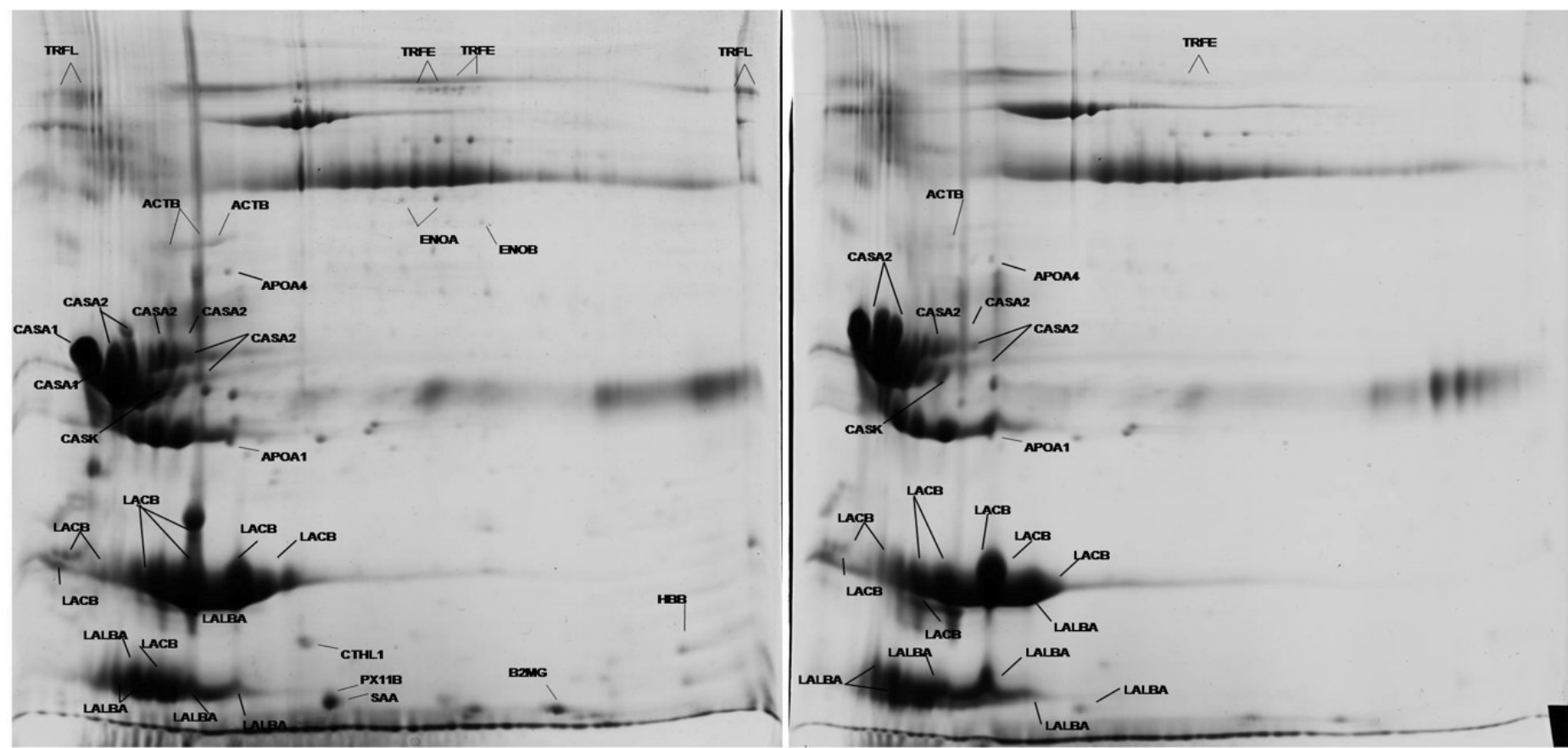
Accession name	Description name	Inoculated side	Non-inoculated side
APOA1_BOVIN	Apolipoprotein A-I	+	+
APOA4_BOVIN	Apolipoprotein A-IV	+	+
B2MG_SHEEP	Beta-2-microglobulin	+	+
LACB_SHEEP	Beta-lactoglobulin-1/B	+	+
CTHL1_SHEEP	Cathelicidin-1	+	+
CASK_SHEEP	Kappa-casein	+	+
TRFL_CAPHI	Lactotransferrin	+	+
TRFE_BOVIN	Serotransferrin	+	+
ALBU_SHEEP	Serum albumin	+	+
SAA_SHEEP	Serum amyloid A protein	+	+
TFP11_RABIT	Tuftelin-interacting protein 11	+	+

+: protein observed with differential expression in milk sample from respective mammary gland from at least one ewe.

Figure II.18 shows 2-DE gels from milk samples of both sides of the udder of same animal at two different time points.



(b)



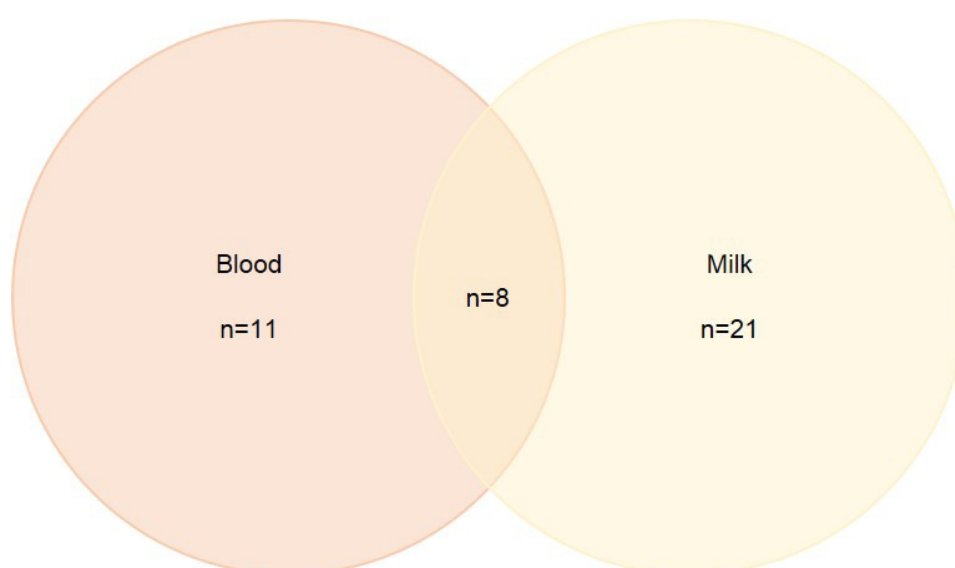
D0: day of inoculation; D2: days after inoculation.  
Details regarding proteins in Tables II.xviii and II.xix.  
Horizontal axis: isoelectric point 3 to 10 (non-linear) from left to right; vertical axis: molecular weight 10 to 100 kDa (non-linear) from bottom to top.

## Comparison of proteomics findings in blood and milk samples of the experimental ewes

### *Findings before inoculation*

In total, eight proteins, namely: alpha-1-antiproteinase, apolipoprotein A-I, apolipoprotein A-IV, fibrinogen beta chain, haemoglobin subunit alpha-1, serotransferrin, serum albumin, transthyretin, were identified concurrently in samples of blood or milk collected before inoculation (Tables II.vi and II.vii, Figure II.19).

**Figure II.19.** Venn diagram of number of proteins that had been identified in the blood or milk sample of the same ewe before deposition of *M. haemolytica* into one teat (protein identification by MALDI-TOF MS).



### *Findings after inoculation*

Of 33 proteins with differential expression in blood after inoculation, 15 also showed differential expression in milk from inoculated side (in total, 89 proteins with differential expression) and 7 in milk from non-inoculated side (in total, 20 proteins with differential expression) of the udder. List of these proteins and the status changes in blood and milk are in Table II.xx and Figure II.20.

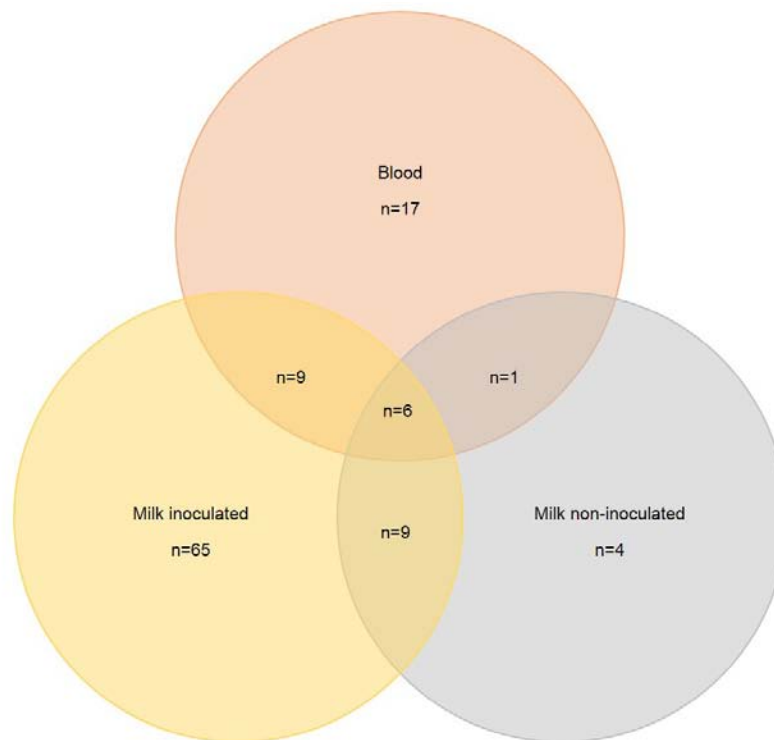


**Table II.xx.** List of proteins that were observed concurrently with downregulation, new expression, upregulation or fluctuation in blood and milk samples from both inoculated and non-inoculated side of the udder, subsequently to inoculation of one teat with *M. haemolytica* in comparison to D0 (protein identification by MALDI-TOF MS).

Accession name	Description name	Blood	Milk	
			inoculated side of udder	non-inoculated side of udder
ACTB_SHEEP	Actin, cytoplasmic 1	↓	↑	-
TFP11_RABIT	Tuftelin-interacting protein 11	↓	↓↑	↑
HBA1_CAPHI	Haemoglobin subunit alpha-1	↑	↓	-
HPT_CAPIB	Haptoglobin	↑	↑	-
MYH1_BOVIN	Myosin-1	↑	↓	-
PRDX6_PIG	Peroxiredoxin-6	↑	↑	-
A1AT_SHEEP	Alpha-1-antiproteinase	↓↑	↓↑	↓↑
APOA1_BOVIN	Apolipoprotein A-I	↓↑	↓↑	↓↑
APOA4_BOVIN	Apolipoprotein A-IV	↓↑	↓↑	↓↑
FIBB_BOVIN	Fibrinogen beta chain	↓↑	↓↑	-
HBB_SHEEP	Haemoglobin subunit beta	↓↑	↑	-
GELS_BOVIN	Gelsolin	↓↑	-	↑
TRFE_BOVIN	Serotransferrin	↓↑	↓↑	↓↑
ALBU_SHEEP	Serum albumin	↓↑	↓↑	↓↑
TTHY_SHEEP	Transthyretin	↓↑	↓↑	-
VTDB_BOVIN	Vitamin D-binding protein	↓↑	↑	-

↓: downregulation after inoculation, ↑: new expression or upregulation after inoculation, ↓↑: fluctuation after inoculation, -: no change after inoculation.

**Figure II.20.** Proteins that were observed with downregulation, new expression, upregulation or fluctuation in blood or milk (from both inoculated or non-inoculated side of the udder) samples, subsequently to inoculation of one teat with *M. haemolytica* (protein identification by MALDI-TOF MS).



In six of above proteins: actin, cytoplasmic 1, fibrinogen beta chain, serotransferrin, serum albumin, transthyretin, tuftelin-interacting protein 11, downregulation in blood and upregulation in milk were observed within 24 hours after challenge.

**Clustering of proteins according to biological process, in which they participated**

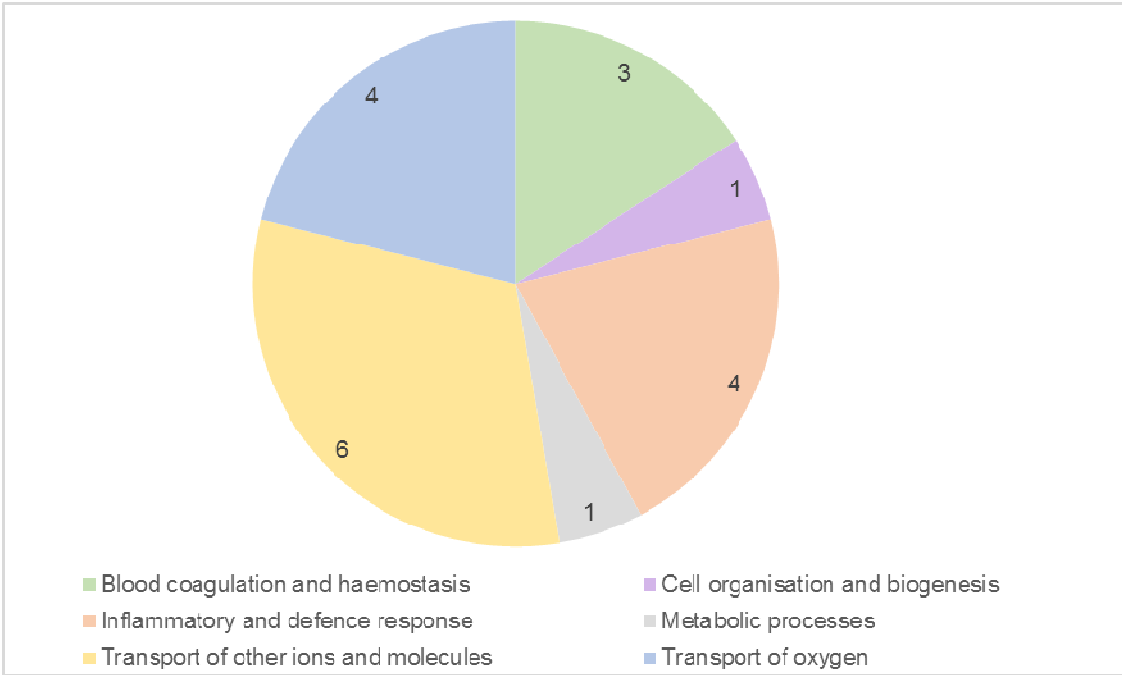
*Proteins identified in samples collected before inoculation of the experimental ewes*

The 19 proteins identified in a blood sample collected from one ewe, before inoculation, could be classified as in Table II.xxi and Figure II.21.

**Table II.xxi.** Biological processes, in which 19 proteins identified in a blood sample collected from a ewe, before deposition of *M. haemolytica* into one teat, were involved (protein identification by MALDI-TOF MS).

Biological process	n	Proteins
Blood coagulation and haemostasis	3	beta-2-glycoprotein 1, fibrinogen gamma-B chain, plasminogen (fragment)
Cell organisation and biogenesis	1	gelsolin
Inflammatory and defence response	4	alpha-1-antiproteinase, fibrinogen beta chain, haptoglobin, serum amyloid A protein
Metabolic processes	1	apolipoprotein A-I
Transport of other ions or molecules	6	apolipoprotein A-IV, ceruloplasmin, retinol-binding protein 4, serotransferrin, serum albumin, transthyretin
Transport of oxygen	4	haemoglobin subunit alpha-1, haemoglobin subunit beta, haemoglobin subunit beta-A, haemoglobin subunit beta-C

**Figure II.21.** Biological processes, in which 19 proteins identified in a blood sample collected from a ewe, before deposition of *M. haemolytica* into one teat, were involved (protein identification by MALDI-TOF MS).

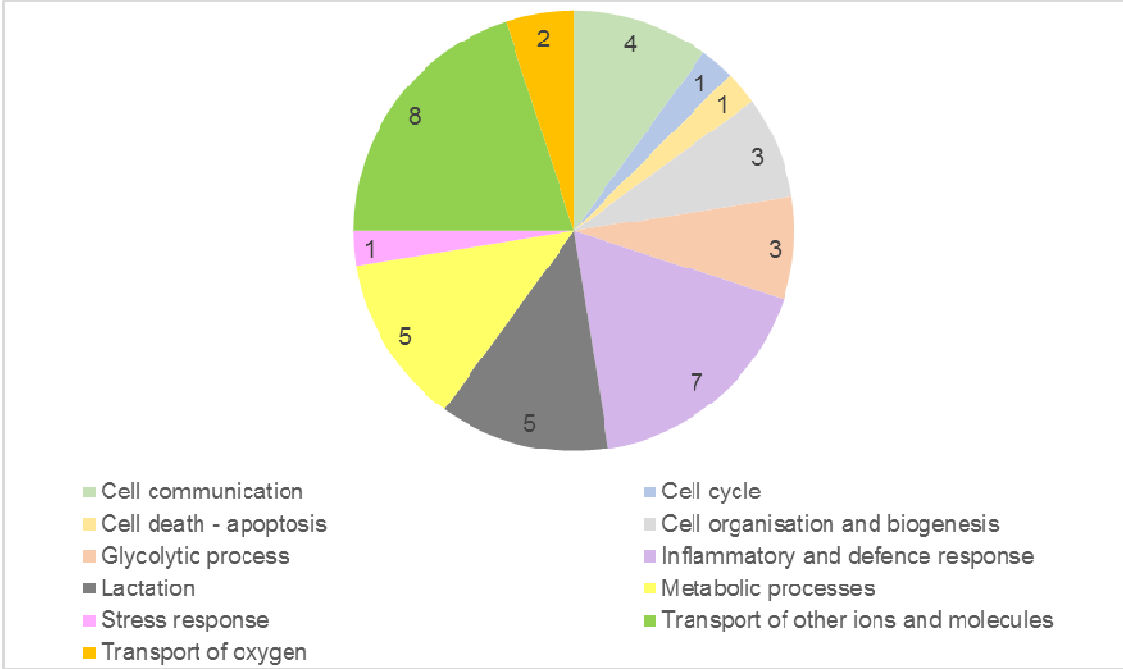


The 40 proteins identified in two milk samples collected from two ewes, before inoculation, could be classified as in Table II.xxii and Figure II.22.

**Table II.xxii.** Biological processes, in which 40 proteins identified in two milk samples collected from each of two ewes, before deposition of *M. haemolytica* into one teat of each animal, were involved (protein identification by MALDI-TOF MS).

Biological process	n	Proteins
Cell communication	4	E3 ubiquitin-protein ligase, myosin-1, myosin-7, rhodopsin kinase
Cell cycle	1	tuftelin-interacting protein 1
Cell death – apoptosis	1	interstitial collagenase
Cell organisation and biogenesis	3	actin, cytoplasmic 1, actin, cytoplasmic-2, keratin, type I cytoskeletal 10
Glycolytic process	3	beta-enolase, fructose-bisphosphate aldolase A, pyruvate kinase
Inflammatory and defence response	7	alpha-1-antiproteinase, alpha-2-HS-glycoprotein, beta-2-microglobulin, fibrinogen beta chain, lactoperoxidase, lactotransferrin, polymeric immunoglobulin receptor
Lactation	5	alpha-lactalbumin, alpha-S1-casein, alpha-S2-casein, beta-lactoglobulin-1/B, kappa-casein
Metabolic processes	5	apolipoprotein A-I, carbonic anhydrase 3, glycogen phosphorylase, muscle form, L-lactate dehydrogenase A chain, neprilysin
Stress response	1	heat shock cognate 71 kDa protein
Transport of other ions and molecules	8	apolipoprotein A-IV, ATP synthase subunit beta, mitochondrial, beta-casein, fatty acid-binding protein, heart, serotransferrin, serum albumin, transthyretin, vitamin D-binding protein
Transport of oxygen	2	haemoglobin subunit alpha-1, haemoglobin subunit epsilon-1

**Figure II.22.** Biological processes, in which 40 proteins identified in two milk samples collected from two ewes, before deposition of *M. haemolytica* into one teat of each animal, were involved (protein identification by MALDI-TOF MS).



*Proteins identified in blood samples collected after inoculation of the experimental ewes*

The 33 proteins differentially expressed in blood samples collected from five ewes, after inoculation, could be classified as in Table II.xxiii and Figure II.23.

**Table II.xxiii.** Biological processes, in which 33 proteins differentially expressed in blood samples collected from five ewes, after deposition of *M. haemolytica* into one teat of each animal, were involved (protein identification by MALDI-TOF MS).

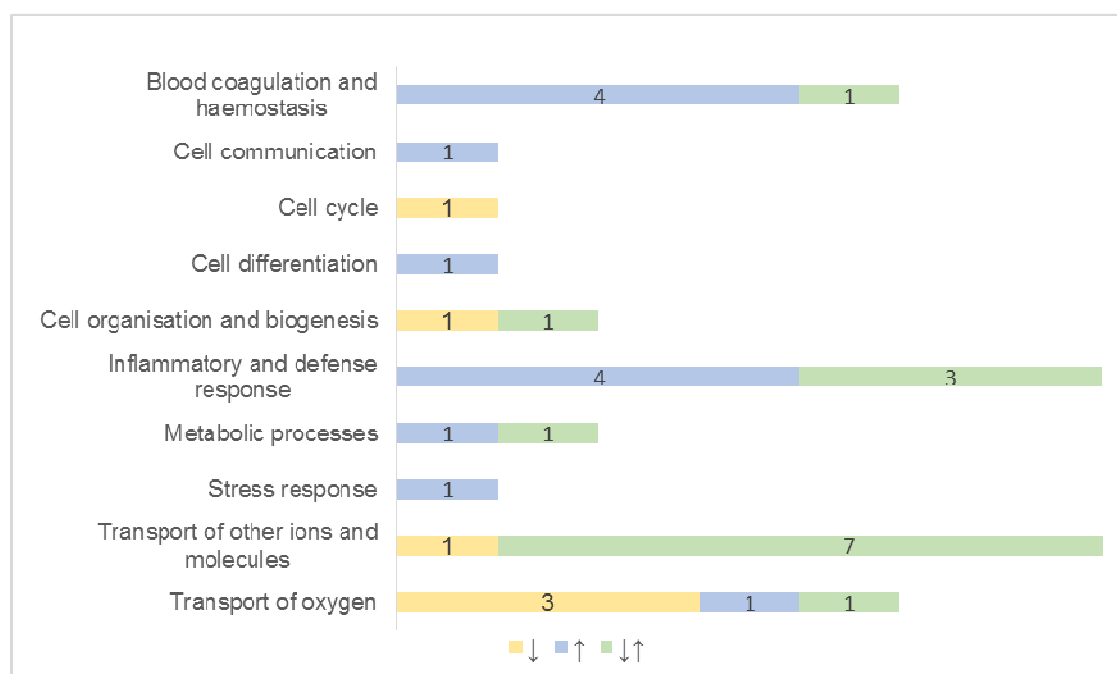
Biological process		n	Proteins
Blood coagulation and haemostasis	↑	4	angiotensinogen, antithrombin-III, beta-2-glycoprotein 1, fibrinogen gamma-B chain
	↓↑	1	plasminogen (fragment)
Cell communication	↑	1	myosin-1
Cell cycle	↓	1	tuftelin-interacting protein 1
Cell differentiation	↑	1	ETS-related transcription factor Elf-5
Cell organisation and biogenesis	↓	1	actin, cytoplasmic 1
	↓↑	1	gelsolin

**Table II.xxiii.** (continued).

Biological process		n	Proteins
Inflammatory and defence response	↑	4	complement C3, complement factor B, haptoglobin, interleucin-4
	↓↑	3	alpha-1-antiproteinase, alpha-2-macroglobulin, fibrinogen beta chain
Metabolic processes	↑	1	peroxiredoxin-6
	↓↑	1	apolipoprotein A-I
Stress response	↑	1	cysteine and histidine-rich domain-containing protein 1
Transport of other ions and molecules	↓	1	haemopexin
	↓↑	7	apolipoprotein A-IV, ceruloplasmin, retinol-binding protein 4, serotransferrin, serum albumin, transthyretin, vitamin D-binding protein
	↓	3	haemoglobin subunit alpha-1/2, haemoglobin subunit beta-A, haemoglobin subunit beta-C
Transport of oxygen	↑	1	haemoglobin subunit alpha-1
	↓↑	1	haemoglobin subunit beta

↓: downregulation after inoculation, ↑: new expression or upregulation after inoculation, ↓↑: fluctuation after inoculation, -: no change after inoculation.

**Figure II.23.** Biological processes, in which 33 proteins differentially expressed in blood samples collected from five ewes, after deposition of *M. haemolytica* into one teat of each animal, were involved (protein identification by MALDI-TOF MS).



↓: downregulation after inoculation, ↑: new expression or upregulation after inoculation, ↓↑: fluctuation after inoculation, -: no change after inoculation.

#### *Proteins identified in milk samples collected after inoculation of the experimental ewes*

The 65 proteins identified in a milk sample collected from one ewe, 12 h after inoculation, could be classified as in Table II.xxiv and Figure II.24.

**Table II.xxiv.** Biological processes, in which 65 proteins identified in a milk sample collected from a ewe, 12 h after deposition of *M. haemolytica* into the ipsilateral teat, were involved (protein identification by MALDI-TOF MS).

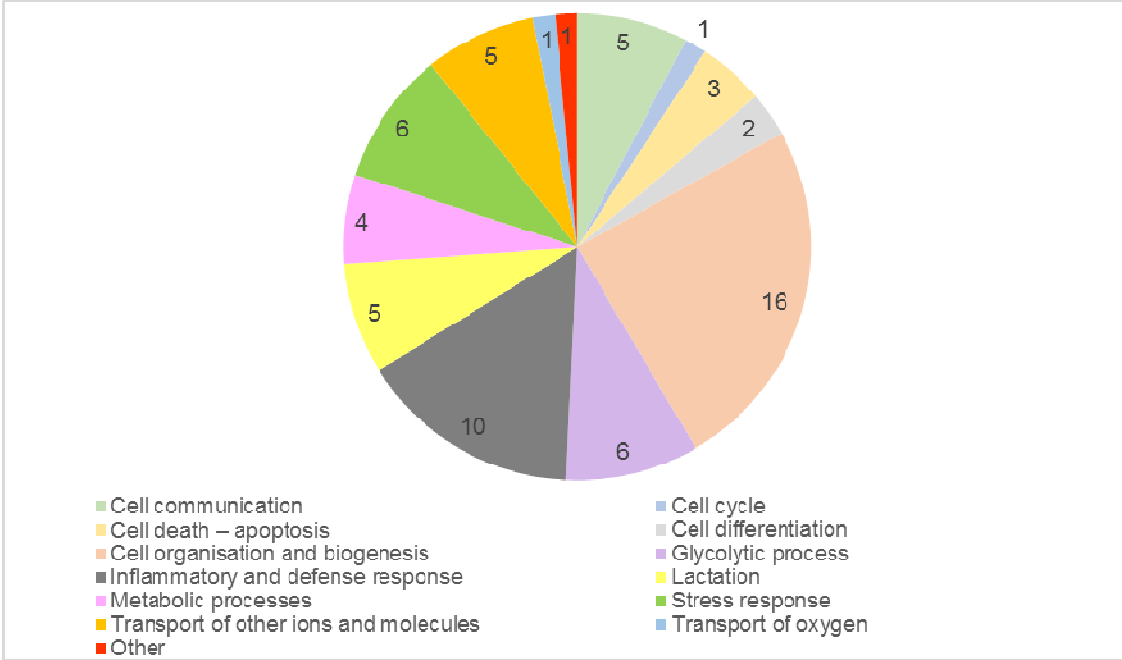
Biological process	n	Proteins
Cell communication	5	78 kDa glucose-regulated protein, phosphatidylethanolamine-binding protein 1, T-complex protein 1 subunit beta, tropomyosin alpha-3 chain, vinculin
Cell cycle	1	tuftelin-interacting protein 11
Cell death – apoptosis	3	cathelin-related peptide SC5, glyceraldehyde-3-phosphate dehydrogenase (fragment), interstitial collagenase

**Table II.xxiv.** (continued).

Biological process	n	Proteins
Cell differentiation	2	nucleoside diphosphate kinase A, phosphoglycerate kinase 1
Cell organisation and biogenesis	16	actin, cytoplasmic 1, actin, cytoplasmic 2, brain-specific angiogenesis inhibitor 1-associated protein 2, cofilin-1, ezrin, F-actin-capping protein subunit alpha-1, keratin, type I cytoskeletal 10, tubulin alpha-1B chain, tubulin alpha-1D chain, tubulin alpha-3 chain, tubulin alpha-4A chain, tubulin beta chain, tubulin beta-2B chain, tubulin beta-4B chain, tubulin beta-5 chain, tubulin beta-6 chain
Glycolytic process	6	alpha-enolase, beta-enolase, isocitrate dehydrogenase (NADP) cytoplasmic, phosphoglycerate mutase 1, phosphomannomutase 2, triosephosphate isomerase
Inflammatory and defence response	10	beta-2-microglobulin, cathelicidin-1, cathelicidin-2, fibrinogen beta chain, haptoglobin, lactoperoxidase, lactotransferrin, protein S100-A9, serum amyloid A protein, transforming growth factor beta-1
Lactation	5	alpha-lactalbumin, alpha-S1-casein, alpha-S2-casein, beta-lactoglobulin-1/B, kappa-casein
Metabolic processes	4	apolipoprotein A-I, glutathione S-transferase P, nucleoside diphosphate kinase B, peroxiredoxin-6
Stress response	6	heat shock cognate 71 kDa protein, heat shock protein beta-1, heat shock protein HSP 90-alpha, heat shock protein HSP 90-beta, protein disulfide-isomerase, protein disulfide-isomerase A3
Transport of other ions and molecules	5	alpha-actinin-4, apolipoprotein A-IV, serotransferrin, serum albumin, vitamin D-binding protein
Transport of oxygen	1	haemoglobin subunit beta
Other	1	ribonuclease



**Figure II.24.** Biological processes, in which 65 proteins identified in a milk sample collected from a ewe, 12 h after deposition of *M. haemolytica* into the ipsilateral teat, were involved (protein identification by MALDI-TOF MS).



The 89 proteins differentially expressed in milk samples collected from the inoculated side of the udder of five ewes, after inoculation, could be classified as in Table II.xxv and Figure II.25.

**Table II.xxv.** Biological processes, in which 89 proteins differentially expressed in milk samples collected from five ewes, after deposition of *M. haemolytica* into the ipsilateral teat of each animal, were involved (protein identification by MALDI-TOF MS).

Biological process		n	Proteins
Cell communication	↓	3	E3 ubiquitin-protein ligase BRE1A, myosin-1, rhodopsin kinase
	↑	6	78 kDa glucose-regulated protein, myosin-7, phosphatidylethanolamine-binding protein 1, T-complex protein 1 subunit beta, tropomyosin alpha-3 chain, vinculin
Cell cycle	↓↑	1	tuftelin-interacting protein 11
Cell death – apoptosis	↑	4	caspase-1, cathelin-related peptide SC5, chitinase-3-like protein 1, glyceraldehyde-3-phosphate dehydrogenase (fragment)
	↓↑	1	interstitial collagenase

**Table II.xxv.** (continued).

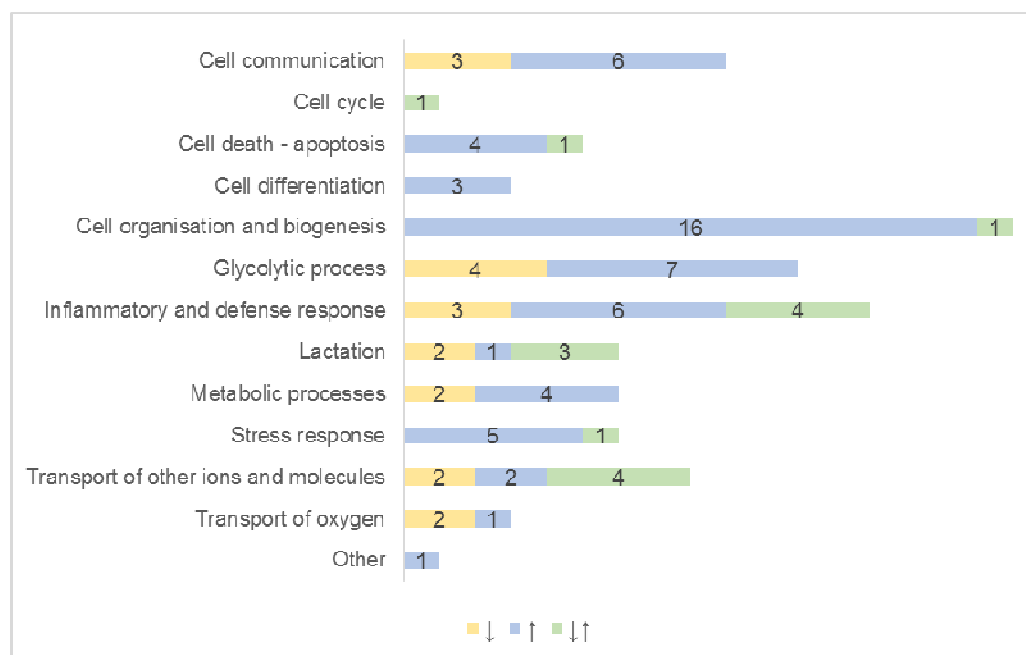
Biological process		n	Proteins
Cell differentiation	↑	3	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1, nucleoside diphosphate kinase A, phosphoglycerate kinase 1
Cell organisation and biogenesis	↑	16	actin, cytoplasmic 1, brain-specific angiogenesis inhibitor 1-associated protein 2, cofilin-1, ezrin, F-actin-capping protein subunit alpha-1, peroxisomal membrane protein 11B, phakinin, tubulin alpha-1B chain, tubulin alpha-1D chain, tubulin beta-2B chain, tubulin alpha-3 chain, tubulin alpha-4A chain, tubulin beta chain, tubulin beta-4B chain, tubulin beta-5 chain, tubulin beta-6 chain
	↓↑	1	actin, cytoplasmic 2
Glycolytic process	↓	4	fructose-bisphosphate aldolase A, glycogen phosphorylase, muscle form, L-lactate dehydrogenase A chain, pyruvate kinase alpha-enolase, beta-enolase, galactose-3-O-sulfotransferase 3, isocitrate dehydrogenase (NADP) cytoplasmic, phosphoglycerate mutase 1, phosphomannomutase 2, triosephosphate isomerase
	↑	7	
Inflammatory and defence response	↓	3	alpha-1-antiproteinase, alpha-2-HS-glycoprotein, lactoperoxidase
	↑	6	cathelicidin-1, cathelicidin-2, haptoglobin, S100-A9 protein, serum amyloid A, transforming growth factor beta-1
	↓↑	4	beta-2-microglobulin, fibrinogen beta chain, lactotransferrin, polymeric immunoglobulin receptor
Lactation	↓	2	beta-casein, beta-lactoglobulin-1/B
	↑	1	kappa-casein
	↓↑	3	alpha-lactalbumin, alpha-S1-casein, alpha-S2-casein
Metabolic processes	↓	2	carbonic anhydrase 3, neprilysin
	↑	4	apolipoprotein A-I, glutathione S-transferase P, nucleoside diphosphate kinase B, peroxiredoxin-6
Stress response	↑	5	heat shock protein beta-1, heat shock protein HSP 90-alpha, heat shock protein HSP 90-beta, protein disulfide-isomerase, protein disulfide-isomerase A3
	↓↑	1	heat shock cognate 71 kDa protein

**Table II.xxv.** (continued).

Biological process		n	Proteins
Transport of other ions and molecules	↓	2	ATP synthase subunit beta mitochondrial, fatty acid-binding protein, heart,
	↑	2	alpha-actinin-4, vitamin D-binding protein
	↓↑	4	apolipoprotein A-IV, serotransferrin, serum albumin, transthyretin
Transport of oxygen	↓	2	haemoglobin subunit alpha-1, haemoglobin subunit epsilon-1
	↑	1	haemoglobin subunit beta
Other	↑	1	ribonuclease

↓: downregulation after inoculation, ↑: new expression or upregulation after inoculation, ↓↑: fluctuation after inoculation, -: no change after inoculation.

**Figure II.25.** Biological processes, in which 89 proteins differentially expressed in milk samples collected from five ewes, after deposition of *M. haemolytica* into the ipsilateral teat of each animal, were involved (protein identification by MALDI-TOF MS).



↓: downregulation after inoculation, ↑: new expression or upregulation after inoculation, ↓↑: fluctuation after inoculation, -: no change after inoculation.

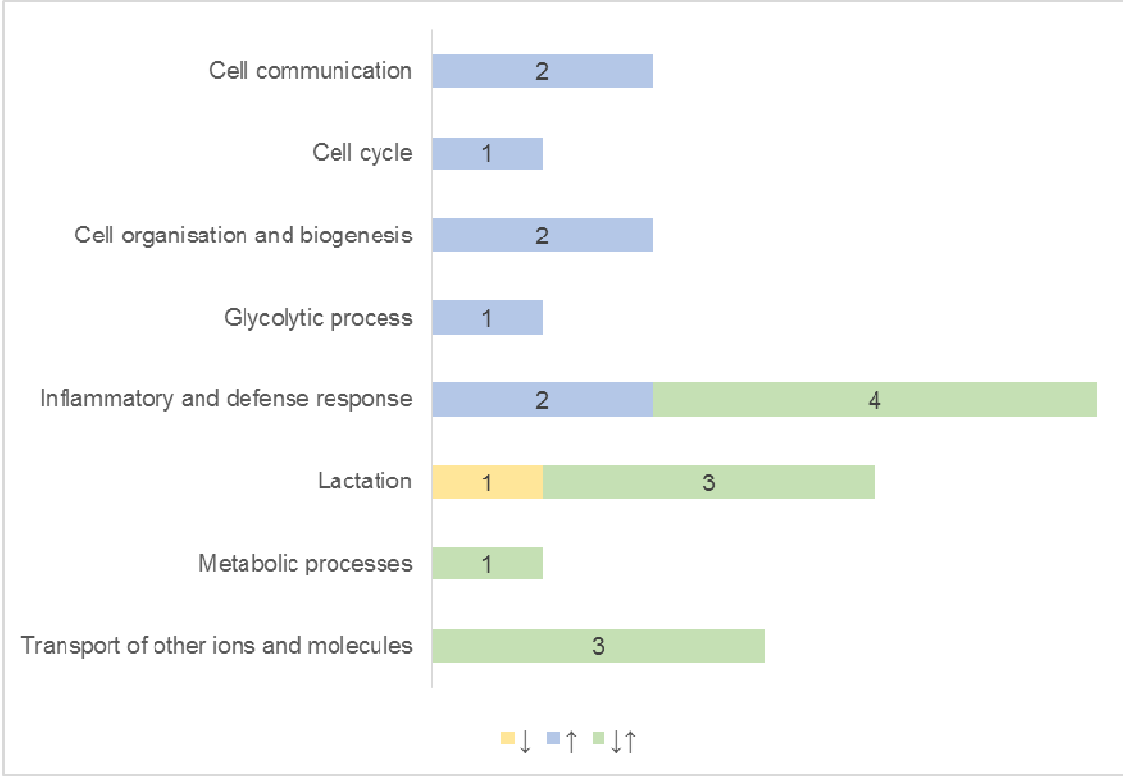
The 20 proteins differentially expressed in milk samples collected from the contralateral to the inoculated side of the udder of five ewes, after inoculation, could be classified as in Table II.xxvi and Figure II.26.

**Table II.xxvi.** Biological processes, in which 20 proteins differentially expressed in milk samples collected from five ewes, after deposition of *M. haemolytica* into the contralateral teat of each animal, were involved (protein identification by MALDI-TOF MS).

Biological process		n	Proteins
Cell communication	↑	2	myosin-4, nectin-1
Cell cycle	↑	1	tuftelin-interacting protein 11
Cell organisation and biogenesis	↑	2	alpha-tubulin N-acetyltransferase 1, gelsolin
Glycolytic process	↑	1	phosphoglucosmutase-1
Inflammatory and defence response	↑	2	cathelicidin-1, serum amyloid A protein
	↓↑	4	alpha-1-antiproteinase, alpha-2-HS-glycoprotein, beta-2-microglobulin, serotransferrin
Lactation	↓	1	alpha-lactalbumin
	↓↑	3	alpha-S2-casein, beta-lactoglobulin-1/B, kappa-casein
Metabolic processes	↓↑	1	apolipoprotein A-I
Transport of other ions and molecules	↓↑	3	apolipoprotein A-IV, serotransferrin, serum albumin

↓: downregulation after inoculation, ↑: new expression or upregulation after inoculation, ↓↑: fluctuation after inoculation, -: no change after inoculation.

**Figure II.26.** Biological processes, in which 20 proteins differentially expressed in milk samples collected from five ewes, after deposition of *M. haemolytica* into the contralateral teat of each animal, were involved (protein identification by MALDI-TOF MS).



↓: downregulation after inoculation, ↑: new expression or upregulation after inoculation, ↓↑: fluctuation after inoculation, -: no change after inoculation.

## **CHAPTER III**

# **SIGNIFICANCE OF CATHELICIDIN-1 IN MILK OF EWES WITH INDUCED *MANNHEIMIA* *HAEMOLYTICA* MAMMARY INFECTION**

## A. INTRODUCTION

Cathelicidins, together with defensins, belong to the large group of cationic peptides with amphipathic properties and are part of the immune system in many vertebrates, including humans and domestic animals (Zanetti et al. 1995, Selsted and Ouellette 2005). The existence of a family of peptides with antimicrobial properties termed 'cathelicidins' was established based on the presence of a conserved cathelin domain. Identification of the first cathelicidin, namely cecropin, has been reported in 1980 (Hultmark et al. 1980); the protein was identified in tissues of the Saturniidae moth *Hyalophora cecropia* during an investigation into insect immunity (Hultmark et al. 1980). Identification of another member of the family, namely magainin, has been reported in 1987 (Zasloff 1987); this was identified in the skin of the African clawed frog *Xenopus leavis* (Zasloff 1987). Then, in the late 1980's, bactenecins have been identified in bovine neutrophils (Gennaro et al. 1989).

In mammals, the peptide family termed 'cathelicidins', having a common proregion (cathelin domain), was first identified in mammals in bone marrow myeloid cells (Zanetti et al. 1995, Bals and Wilson 2003). Hence, they are also termed 'myeloid antimicrobial peptides'. Myeloid cells (e.g., neutrophils) are the predominant source of cathelicidins in mammals, although expression of these proteins has also been detected in non-myeloid cells, e.g. expression of cathelin-related antimicrobial peptide has been identified in mammary epithelial cells of mice (Zanetti 2005) and expression of human cationic antimicrobial protein 18 has been identified in lung epithelium (Bals et al. 1998) or squamous epithelia of skin (Frohm Nilsson et al. 1999).

Various cathelicidins thereafter been identified in several vertebrate animals, including pigs (Kokryakov et al. 1993), goats (Shamova et al. 1999), horses (Scocchi et al. 1999), sheep (Brogden et al. 2001), various fish species (Uzzell et al. 2003), humans (Dürr et al. 2006), buffaloes (Das et al. 2006), chickens (Xiao et al. 2006), dogs (Sang et al. 2007), deer (Fernandez de Mera et al. 2008), the banded krait snakes *Bungarus fasciatus* (Wang et al. 2008) and cats (Leonard et al. 2011). Moreover, peptides leading to potential identification of cathelicidins have been found in guinea pigs (Nagaoka et al. 1997), mice (Gallo et al. 1997), rhesus monkeys (Bals et al. 2001, Zhao et al. 2001) and rats (Termen et al. 2003).

Cathelicidins have been named after the region with increased homology to cathelin, the cathepsin L inhibitor, at their N-terminal. The 'proregion' 'cathelin' domain shows substantial inter-species homology in cathelin basic amino acid sequence, whereas the C-terminus domain (the

antimicrobial domain) encoding the mature peptide (with potent antimicrobial activity) shows high inter- and intra-species diversity (Boman 1995, Zanetti et al. 1995, 2005, Bals and Wilson 2003).

Cathelicidins are considered to be stored into the secondary secretory granules of neutrophils, as well as in monocytes/macrophages of humans as inactive precursors ('pre-propeptides') (Zanetti et al. 1991, 1995, Zanetti 2005). They can be released as mature peptides extracellularly, upon leucocyte activation, after being cleaved by neutrophil elastase (Treffers et al. 2005).

The C-terminal domains in some cathelicidin peptides are  $\alpha$ -helical, in others  $\beta$ -hairpin and in others proline/arginine rich. The mature peptide ranges in size from 12 to 80 or more amino acid residues (Zanetti et al. 1995). The cathelicidin family consists of five distinct groups of peptides (Zanetti 2005). These comprise: (i) cyclic dodecapeptides with one disulfide bond, (ii) porcine protegrins with two disulfide bonds, (iii) peptides with  $\alpha$ -helical structure, e.g. bovine BMAP-27, BMAP-28 and BMAP-34, ovine SMAP-29 and SMAP-34, porcine PMAP-23, PMAP-36 and PMAP-37, human hCAP18 and equine CATH-1, CATH-2 and CATH-3, (iv) peptides with increased number of tryptophan residues, e.g., indolicidin, and peptides with increased number of proline and arginine residues and (v) short molecules arranged in tandem repeats, e.g., as bactenecins (bovine Bac5 and bovine 7, ovine OaBac5 and OaBac7.5, porcine PR-39, prophenins) (Tossi et al. 2000, Tomasinsig and Zanetti 2005, Zanetti 2005).

All cathelicidins are encoded by genes consisting of four exons. The first exon covers a sequence encoding the signal peptide (part pre-) of 29 to 30 amino acid residues in size. Exons 2 and 3 encode the cathelin domain (part pro-) of 99 to 114 amino acids. Exon 4 encodes the mature peptide, with the antimicrobial domain consisting of 12 to 100 amino acids. In cattle, sheep, pig and chicken chromosomes, all cathelicidin genes have been found to form clusters (Xiao et al. 2006). These have been found located on chromosome 19 in sheep (8 genes), on chromosome 13 in pig and on chromosome 2p at the proximal end as a dense cluster within a 7.5-kb (3 genes) in chickens (Xiao et al. 2006). In cattle, over 10 cathelicidin genes have been found located in the same region on chromosome 22q24 (Scocchi et al. 1997). In human or murine genome, cathelicidin genes have been found located on chromosomes 3 or 9, respectively (Strukelj et al. 1995). Phylogenetic analysis has shown that genes encoding chicken cathelicidins and mammalian neutrophilic granule peptides are probably descendants of a single, remotely related gene that had evolved prior to separation of birds from mammals. However, it is supposed that genes encoding other 'classic' mammalian cathelicidins (cathelicidins with high homology in cathelin domain) may have been duplicated from the ancestral neutrophilic granule peptide gene after mammals and birds drifted apart (Xiao et al. 2006).



There are some studies describing identification of cathelicidins in sheep and goats. Cathelicidins have been implicated in the control in respiratory infections (Broghden et al. 2001) and the elucidation of physiology of ovine trophoblasts (Coyle et al. 2016). Anderson et al. (2004) have found that, in sheep, cathelicidins (SMAP29, OaBac5mini, OaBac7.5mini) had a confirmed activity against Gram-negative and Gram-positive bacteria, as well as against *Candida albicans*. In goats, bactenecin-5 (chBac5, cathelicidin-2) and chBac3.4 (cathelicidin-3.4) have been found to exert a strong activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Shamova et al. 1999, 2009). Further, cathelicidin-1 and cathelicidin-3 have been identified in the mammary secretion of goats with mastitis (Olumee-Shabon et al. 2013); further, cathelicidin-2 has been identified in neutrophils of mammary secretion of goats following intramammary challenge with lipopolysaccharide (Zhang, Lai et al. 2014), whilst Addis et al. (2011) have reported that cathelicidin-1 was produced in mammary epithelial cells. All these results potentially indicating a role for cathelicidins in the mammary gland (Tomasinsig et al. 2010).

Further, association of cathelicidin proteins with mastitis in cows has been evaluated in many studies. Using proteomic analysis, cathelicidin-1, cathelicidin-2, cathelicidin-3 and cathelicidin-4 have been identified in mammary secretion of cows with mastitis (Boehmer et al. 2008, Danielsen et al. 2010, Smolenski et al. 2011). They have also been identified under *in vitro* conditions in mammary epithelial cells of cows (Ibeagha-Awemu et al. 2010).

Cathelicidin-1 has been identified as a predominant protein in the milk sample examined from a ewe 12 h after inoculation of one teat with *Mannheimia haemolytica* (protein identification by MALDI-TOF MS) for production of a post-inoculation protein reference map (Table II.xi). The protein was also observed with new expression in milk samples from the inoculated side of the udder, subsequently to inoculation of one teat with *M. haemolytica* in comparison to D0 (Table II.xii).

Objective of the work described in this chapter was the study of the presence of cathelicidin in milk as a potential diagnostic indicator for detection of mastitis in sheep, using *M. haemolytica* and *Staphylococcus chromogenes* infection models.

## **B. DETAILED EVALUATION OF FINDINGS RELATED TO CATHELICIDIN-1 IN EXPERIMENT OF ‘PROTEOMICS ANALYSIS OF BLOOD AND MILK OF EWES WITH INDUCED *MANNHEIMIA HAEMOLYTICA* MAMMARY INFECTION’**

### **Materials and methods**

#### **Source of data**

Detailed data regarding cathelicidin-1 identification, which had been obtained during processing of blood and milk samples in the Experiment ‘Proteomics analysis of blood and milk of ewes with induced *Mannheimia haemolytica* mammary infection’ (Chapter II, C), have been considered again.

#### **Data management**

#### *Evaluation of results*

Initially, results of presence of cathelicidin-1 in blood or milk samples collected from individual ewes (n=5) before or after inoculation, have been evaluated. Spot densities (optical densities) of cathelicidin-1 spots on 2-DE gels, obtained for each ewe, on each sampling point, during examination of scans of gels by using the PDQuest v.8.0 image processing software, were recorded. Spots representing cathelicidin-1 were matched across gels obtained from samples collected from the same ewe throughout the study. Subsequently, means were calculated for results obtained for each animal on each sampling point.

#### *Statistical computations*

The repeated measures mixed effect linear regression model was used to determine whether spot densities changed over the course of the study period. Models were adjusted for repeated measures within animals. Independent variable was day after challenge. Wilcoxon Signed Rank test was performed to evaluate differences in means of spot densities in samples from the inoculated or the contralateral side of the udder.

An electronic data analysis tool was employed (Vassar Stats: Website for Statistical Computation; Lowry 2012, 2015). Significance level was set at  $P \leq 0.05$ .

## Results

### Findings in examinations before challenge of the experimental ewes

Presence of cathelicidin-1 was not evident in any of the six blood samples collected and processed from ewes before inoculation (1 sample collected on D-2, from one ewe, plus 5 samples collected on D0, one from each of the five ewes into the study). Also, presence of the protein was not evident in any of the 12 milk samples from ewes before inoculation (2 samples collected on D-2, each from a different ewe plus 10 samples collected on D0, two from each of the five ewes into the study, of which one from the side of the udder to be inoculated and the other from the contralateral side of the udder).

### Findings in examinations after challenge of the experimental ewes

#### *Findings in blood samples*

Presence of cathelicidin-1 was not evident in any of the 22 blood samples collected and processed from ewes after inoculation (one sample collected from each of the five ewes into the study on D0+12 h, D1, D2, D3 and one sample collected from each of the remaining two ewes into the study on D4).

#### *Findings in milk samples*

Presence of cathelicidin-1 was recorded in samples from 5/5 ewes, specifically in 19/22 samples, from the inoculated side of the udder, starting 12 h (D0+12 h) and until the 4th day (D4) after inoculation. It was also identified in 3/5 ewes, specifically in 5/22 samples, from the contralateral side of the udder, starting on the 1st day (D1) and until the 3rd day (D3) after inoculation.

In milk samples from the inoculated side of the udder of all ewes, mean spot density of cathelicidin-1 increased sharply, starting at the first post-inoculation sampling (D0+12 h) and progressively decreased thereafter. In milk samples from the non-inoculated side of the udder of

all ewes, mean spot density of the protein also increased, starting at the second post-inoculation sampling (D1) and progressively decreased thereafter.

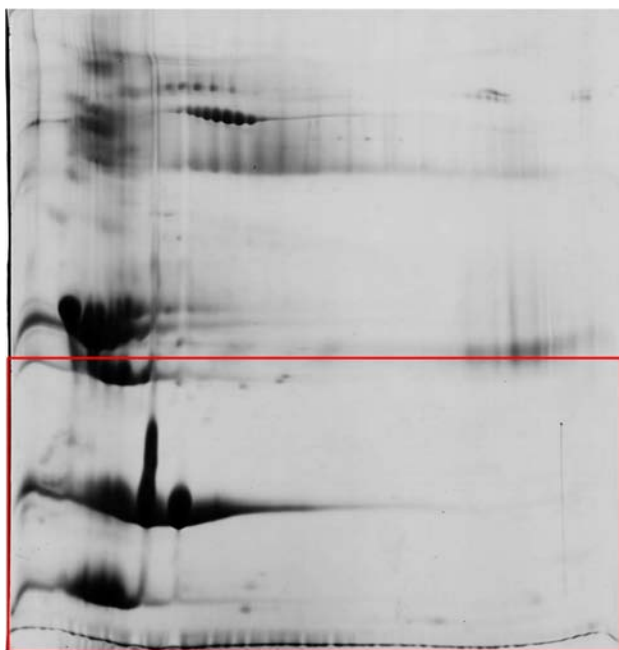
For the inoculated side of the udder, there was clear evidence that results after inoculation were significantly greater than those before inoculation ( $P=0.001$ ), but nevertheless there were no significant differences between results obtained at the various time-points when samples had been collected after challenge ( $P>0.12$ ). For the non-inoculated side of the udder, there were no significant differences neither for results before and after inoculation ( $P=0.085$ ), nor between the various time-points when samples had been collected after challenge ( $P>0.37$ ). Further, differences between results of inoculated and non-inoculated glands were significant ( $P=0.05$ ). Details are in Table III.i and in Figures III.1 and III.2.

**Table III.i.** Spot densities (mean±standard error of the mean) of cathelicidin-1 in 2-DE gels from sequential milk samples from inoculated or non-inoculated side of the udder, subsequently to inoculation of one teat with *M. haemolytica* (protein identification by MALDI-TOF MS).

Udder side	Total spots (n)	Before inoculation	Day after inoculation				
		D0	D0+12 h	D1	D2	D3	D4
Inoculated	2.6±0.7	0.0±0.0	813.1±506.7	637.4±415.6	419.1±283.1	340.4±194.3	786.6±497.4
Non-inoculated	0.8±0.4	0.0±0.0	0.0±0.0	68.2±60.4	14.8±14.8	2.8±2.9	0.0±0.0

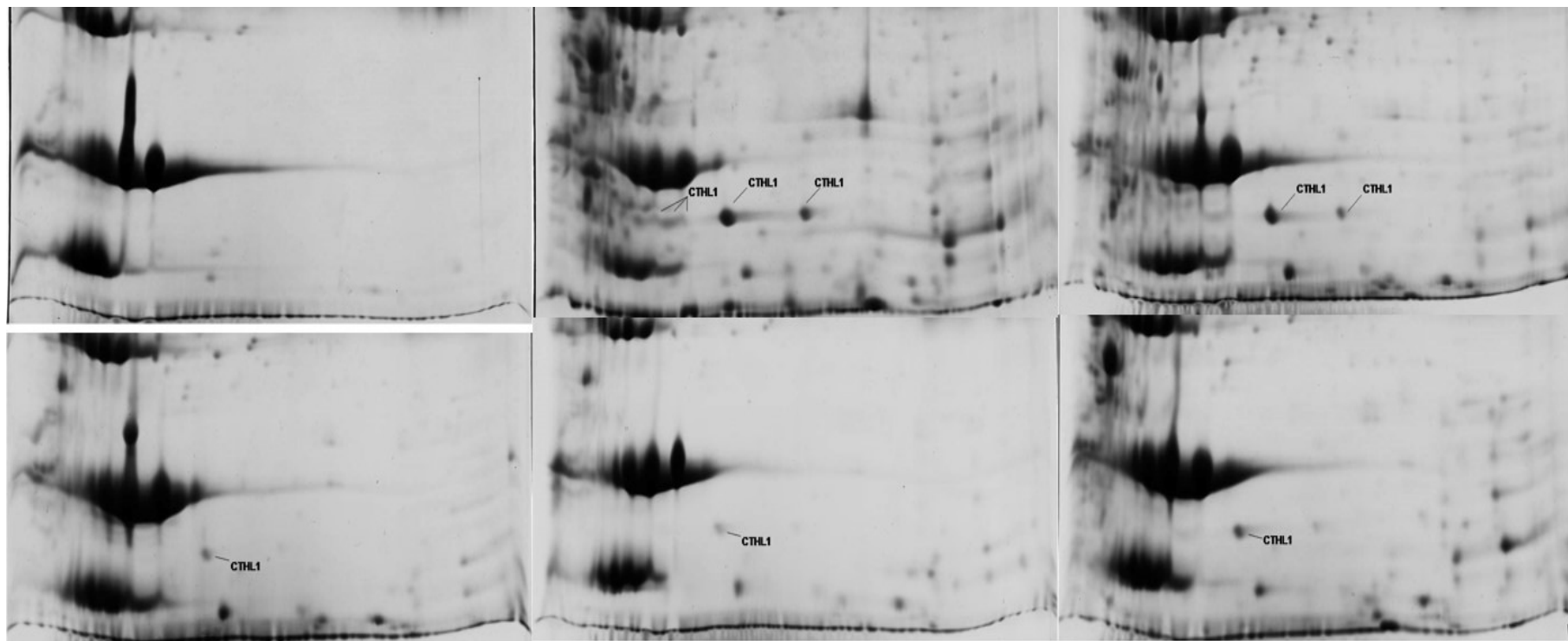
**Figure III.1.** 2-DE gels with annotation of cathelicidin-1, obtained from milk samples collected from the inoculated side of the udder of a ewe before or after inoculation of the ipsilateral teat with *M. haemolytica* (a, b) or from the non-inoculated side of the udder of the same ewe (c) (protein identification by MALDI-TOF MS).

(a) 2-DE gel obtained from milk sample collected before challenge from the inoculated side of the udder of a ewe; area characterised in red indicates the region of the gels shown in detail in (b) and (c).

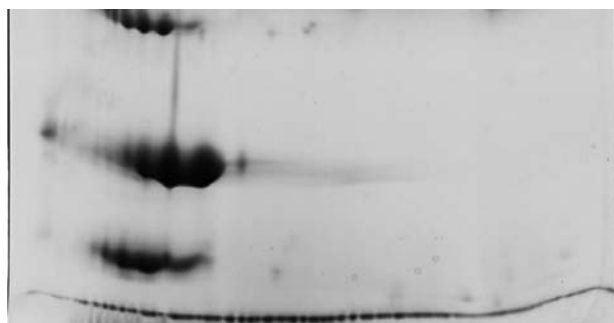


Horizontal axis: isoelectric point 3 to 10 (non-linear) from left to right; vertical axis: molecular weight 10 to 100 kDa (non-linear) from bottom to top.

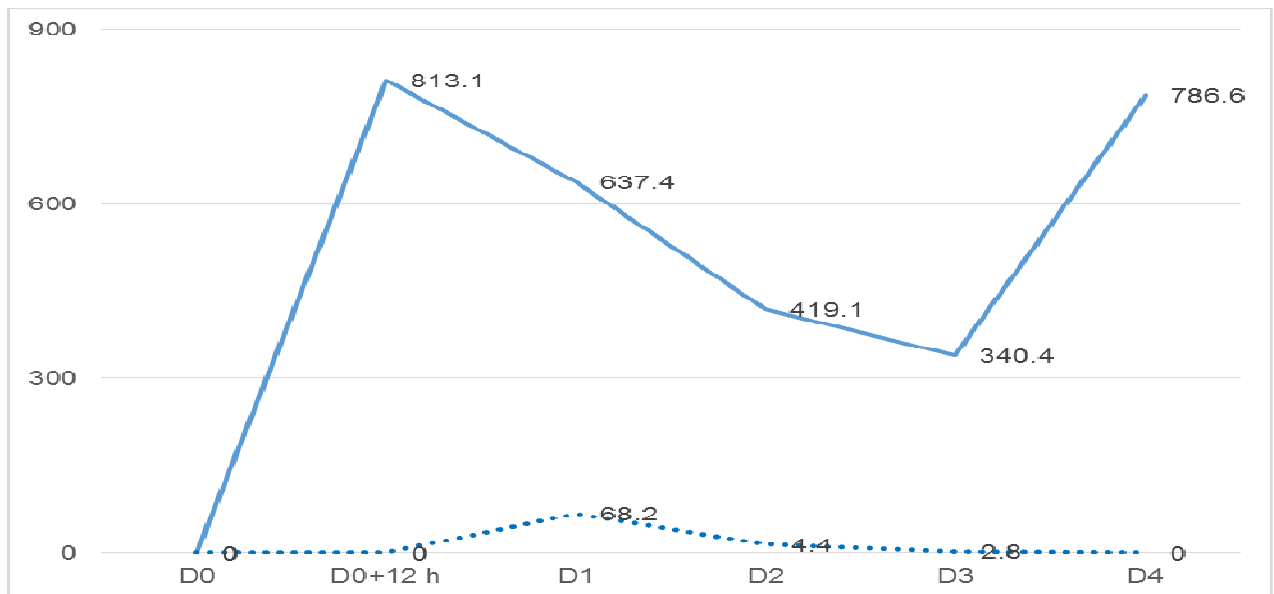
(b) Region of 2-DE gels obtained from milk samples collected sequentially before or after challenge from the inoculated side of the udder of a ewe; from top row to bottom row and from left to right: before inoculation (D0), 12 h after inoculation (D0+12 h), 1 d after inoculation (D1), D2, D3, D4.



(c) Region of 2-DE gel obtained from milk sample collected 12 h after inoculation of the contralateral side of the udder.



**Figure III.2.** Mean spot densities of cathelicidin-1 in 2-DE gels obtained from sequential milk samples from inoculated (straight line) or non-inoculated (dotted line) side of the udder, subsequently to inoculation of one teat with *M. haemolytica* (protein identification by MALDI-TOF MS).



## C. IDENTIFICATION OF CATHELICIDIN-1 IN MILK OF EWES WITH INDUCED MAMMARY INFECTION IN A BLINDED EXPERIMENT

### Materials and methods

#### Experimental overview

Sequentially collected blood and milk samples from 3 Chios-cross ewes were examined during the study. Of the animals, one was inoculated with *Mannheimia haemolytica* and two were inoculated with *Staphylococcus chromogenes*, directly into the gland cistern, on the 6th day after lambing. Development of mastitis had been confirmed by using clinical, bacteriological and cytological techniques. Milk samples were collected sequentially, before and after challenge, for examination by proteomics technologies: two-dimensional polyacrylamide gel electrophoresis and MALDI-TOF mass spectrometry. Proteins were separated in two-dimensional gels from samples; cathelicidin-1 was identified by MALDI-TOF and evaluated. An experimental flow similar to that followed in Experiment 'Proteomics analysis of blood and milk of ewes with induced *Mannheimia haemolytica* mammary infection' was undertaken (Figure II.1).

The clinical phase of the experiment and the conventional laboratory examinations of samples collected during that were performed at the Department of Obstetrics and Reproduction of the University of Thessaly (Ms N.G.C. Vasileiou). Samples were sent for proteomics analysis blinded in relation to details of animals, from which they had been collected.

Conditions prescribed by legislation of the European Union in relation to animal experimentation procedures (Council Directive 86/809/EEC) were met during the experiment from which samples were collected; the experiment was carried out under a licence for experimental procedures obtained from the Greek Ministry of Agriculture.

#### Inoculation procedure

The *Mannheimia haemolytica*, strain VSM08L, was used for inoculation of one ewe; the strain had been used for inoculation of animals in the experiment 'Proteomics analysis of blood and milk of ewes with induced *Mannheimia haemolytica* mammary infection'. A *Staphylococcus chromogenes*, strain 6684, which has been isolated from a case of mastitis in a ewe and is of



known pathogenicity for the mammary gland of this species (Vasileiou et al. 2016), was used for inoculation of two ewes.

Each organism was grown on Columbia blood agar and checked for purity; then it was inoculated into Soy-broth and incubated aerobically at 37 °C for 5 h. Serial dilutions of the broth culture into PBS pH 7.3 were carried out; finally, 0.2 mL of the desired dilution was withdrawn with a syringe. Each inoculum contained 50 to 80 c.f.u. of *M. haemolytica* or  $10^6$  to  $2 \times 10^6$  c.f.u. of *S. chromogenes*, as estimated by the method of Miles and Misra (1938).

The same inoculation methodology as described above (Chapter II, B) has been employed. A notable difference was that sterile plastic fine catheters (Abbocath®) 20 G, 3 cm-long were used and inserted into one teat; that way, the bacterial suspension was deposited directly inside the gland cistern.

## Pre- and post-challenge procedures in the experimental ewes

### *Clinical examination of the udder*

Clinical examination was performed as described above (Chapter II, B) before challenge (D0; D0: day of challenge), as well as 3 h, 6 h, 12 h and 24 h after that.

### *Samplings*

On each occasion, when an examination of the udder was performed, sample collection was carried out. Blood samples were collected into anti-coagulant-treated sterile plastic EDTA-treated tubes. Each sample was mixed by gentle repeated inversions for several seconds to avoid coagulation.

Milk samples were obtained as described above (Chapter II, B). Separate samples were collected from each mammary gland of each experimental animal.

For proteomics examinations, milk samples collected before challenge were processed individually. Milk samples collected from the inoculated side of the udder, after challenge, were also processed individually. However, samples collected from the non-inoculated side of the udder, after challenge, were mixed; each pooled sample consisted of equal volumes of five individual samples (i.e., those collected 3 h, 6 h, 9 h, 12 h and 24 h after inoculation) from the same animal; these were thoroughly mixed, then a final volume of 10 to 15 mL was taken for processing.

## Conventional laboratory examinations in samples from the experimental animals

Haematological examination for leucocyte counts only, as well as bacteriological and cytological examinations of milk samples were performed as described above (Chapter II, B).

Further, the microscopic cell counting method was performed in each milk sample, as described by Fthenakis (1988) and according to the recommendations of the International Dairy Federation (1984). Each milk sample was heated in a water-bath to 35 °C, mixed carefully and then cooled to room temperature. Then, 100 µL of the sample were withdrawn with a calibrated pipette (P200; Gilson, Middleton, USA) and placed on a glass slide on the surface of which an area of 20×5 mm had been marked. The area was filled evenly with milk and the slide was allowed to dry. The milk film was then stained by dipping into a dye solution (details in Appendix, preparation no. 1) for 10 min. Subsequently, the slide was washed and allowed to dry. Cell nuclei (i.e., not cells) were counted.

## Proteomics examinations

All procedures with regard to processing, storage and proteomics evaluation (fractionation, measurement of protein content by Bradford protein assay, first dimension separation, second dimension separation) were performed as described above (Chapter II, B).

During protein identification, as only protein spots of interest were considered those that potentially represented cathelicidin-1, in comparison to results of previous experiment (Chapter II, C). All spots of interest were excised manually by using the methodology described above (Chapter II, B). Further processing of proteins spots of interest was also performed as described above, in order to confirm identification by MALDI-TOF MS (Chapter II, B).

## Data management

### *Definitions*

Clinical mastitis was defined as the presence of clinically evident abnormalities in the mammary gland or the mammary secretion. Subclinical mastitis was defined as the simultaneous isolation of bacteria from milk samples and the detection of positive CMT scores, with no clinically evident abnormalities.

#### *Cytological examinations of milk samples*

In the California Mastitis Test, reactions scored  $\geq 1$  were considered to be indicative of increased cellular content in milk (Fthenakis 1995).

In the microscopic cell counting method, at least 200 cell nuclei were counted in each sample. The number of cell nuclei counted was multiplied by the below working factor to provide number of cells per mL of milk.

- Working factor:  $(20/d) \times (100/b)$  (d: diameter of the field of the microscope, b: number of stripes counted).

#### *Total protein content in milk whey samples, as measured by the Bradford protein assay*

This was calculated as described above (Chapter II, B).

#### *Evaluation of 2-DE gels for identification of protein spots of interest*

Gels were initially evaluated visually after placing on a light source. Only protein spots that were considered to represent cathelicidin-1, in comparison to results of previous experiment (Chapter II, C), were considered as spots of interest and studied.

Comparisons between gels was performed as described above (Chapter II, B). The PDQuest v.8.0 image processing software was used for evaluation of scans of the gels, as described above (Chapter II, B).

#### *Peptide matching and protein searches*

This procedure was applied as described above (Chapter II, B).

#### *Presentation of results of protein identification*

Spot densities obtained from PDQuest v.8.0 for each spot of interest on each gel from sample collected on D0 or after challenge, were recorded. In case of multiple spots indicative of cathelicidin-1, densities of all spots were taken into account.

#### *Evaluation of results*

Initially, results of presence of cathelicidin-1 in milk samples collected from individual ewes (n=3) before or after inoculation, have been evaluated. Spot densities (optical densities) of cathelicidin-1 spots on 2-DE gels, obtained for each ewe, on each sampling point, during examination of scans of gels by using the PDQuest v.8.0 image processing software, were recorded. Spots representing cathelicidin-1 were matched across gels obtained from samples

collected from the same ewe throughout the study. Subsequently, means were calculated for results obtained for each animal on each sampling point.

#### *Statistical computations*

All data were entered into Excel spreadsheets. Descriptive statistics for all parameters were performed.

The repeated measures mixed effect linear regression model was used to determine whether spot densities changed over the course of the study period. Models were adjusted for repeated measures within animals. Independent variable was day after challenge. Wilcoxon Signed Rank test was performed to evaluate differences in means of spot densities in samples from the inoculated or the contralateral side of the udder.

An electronic data analysis tool was employed (Vassar Stats: Website for Statistical Computation; Lowry 2012, 2015). Significance level was set at  $P \leq 0.05$ .

### **Appendix: Details of preparations used in the study**

#### *Preparation no. 1*

Dye solution: 0.6 g methylene blue (Merck Millipore), 54 mL ethanol-96% (Sigma-Aldrich), 40 mL 1.1.1 trichloroethane (Sigma-Aldrich) and 6 mL glacial acetic acid (Sigma-Aldrich).

## **Results**

### **Results of clinical and conventional laboratory examinations in samples from the experimental animals**

#### *Findings in examinations before challenge of the experimental ewes*

The mammary glands of all ewes were clinically healthy in the examination performed before challenge. No bacteria were isolated from any milk sample obtained. California Mastitis Test scores in milk samples were negative (scores 'negative' or 'trace') and somatic cell counts were  $< 0.45 \times 10^6$  cells mL<sup>-1</sup>. Observation of Giemsa-stained milk films revealed only scarce presence of macrophages therein (on average, one cell per 10 fields with the 10× objective lens).

Haematological parameters were within the reference ranges (Kramer 2000, Martin and Aitken 2000, Roger 2008). Detailed results are in Tables III.ii and III.iii.

*Clinical, bacteriological and cytological findings in milk samples after challenge of the experimental ewes*

The ewe inoculated with *M. haemolytica* developed signs of clinical mastitis, whilst the two ewes inoculated with *S. chromogenes* developed subclinical mastitis, i.e., in total, 3/3 animals developed mastitis. *M. haemolytica* or *S. chromogenes* was isolated in pure culture from mammary secretion samples of ewes inoculated with the respective organism (in total, 5/5 *M. haemolytica* and 10/10 *S. chromogenes* isolations), starting 3 h and until 24 h post-inoculation. The CMT increased (>'1') in all ewes (10/15 positive samples). Somatic cell counts increased after inoculation; they were  $<1.0 \times 10^6$  cells mL<sup>-1</sup> 3 h and 6 h post-inoculation and  $>1.0 \times 10^6$  cells mL<sup>-1</sup> thereafter. Leucocytes were seen in Giemsa-stained secretion films; their great majority ( $\geq 90\%$ ) consisted of neutrophils, with a few macrophages and lymphocytes also present. Detailed results are in Table III.ii.

**Table III.ii.** Clinical findings, sequential isolation of *M. haemolytica* or *S. chromogenes* and results of cytological examinations in ewes with one mammary gland inoculated with either organism

(a) Ewe inoculated with *M. haemolytica*.

	Before inoculation	After inoculation				
	D0	3 h	6 h	9 h	12 h	24 h
Clinical findings						
Presence	0/1	0/1	1/1	1/1	1/1	1/1
Isolation of <i>M. haemolytica</i>						
Milk	0/1	1/1	1/1	1/1	1/1	1/1
California Mastitis Test results						
Positive scores	0/1	0/1	1/1	1/1	1/1	1/1
Somatic cell counts						
Values (cells mL <sup>-1</sup> )	$0.430 \times 10^6$	$0.655 \times 10^6$	$1.183 \times 10^6$	$2.784 \times 10^6$	$3.437 \times 10^6$	$2.056 \times 10^6$

**Table III.ii.** (continued).(b) Two ewes inoculation of *S. chromogenes*.

	Before inoculation	After inoculation				
	D0	3 h	6 h	9 h	12 h	24 h
Clinical findings						
Presence	0/2	0/2	0/2	0/2	0/2	0/2
Isolation of <i>S. chromogenes</i>						
Milk	0/2	2/2	2/2	2/2	2/2	2/2
California Mastitis Test results						
Positive scores	0/2	0/2	0/2	2/2	2/2	2/2
Somatic cell counts						
Values	$0.421 \times 10^6$	$0.721 \times 10^6$	$0.939 \times 10^6$	$2.752 \times 10^6$	$2.227 \times 10^6$	$1.954 \times 10^6$
(cells mL <sup>-1</sup> )	$0.372 \times 10^6$	$0.647 \times 10^6$	$0.850 \times 10^6$	$1.980 \times 10^6$	$3.030 \times 10^6$	$3.212 \times 10^6$

D0: day of inoculation; D1, D2, D3, D4: days after inoculation

n/m: positive results out of total animals sampled

None of the contralateral side of the udder of the experimental ewes developed clinical or subclinical mastitis ( $P=0.007$  versus the inoculated sides). No bacteria were isolated from any sample from these sides (in total, 0/5 and 0/10 isolations for the two organisms, respectively,  $P<0.001$  versus the inoculated sides). No increased CMT scores were recorded in any milk sample from the non-inoculated sides of the udder of the experimental ewes (in total, 0/15 samples with increased CMT score,  $P<0.001$  versus the inoculated sides). Somatic cell counts remained  $<0.45 \times 10^6$  cells mL<sup>-1</sup> in all samples during the study ( $P<0.04$  versus the inoculated sides).

#### *Haematological findings after challenge of the experimental ewes*

After challenge, there was a significant increase in total leucocyte counts, which started already 9 hours after challenge ( $0.07<P<0.45$ , compared to values before challenge, depending on sampling point). Increase in leucocyte counts was characterised primarily by increased counts of neutrophil and immature neutrophil counts ( $>3$ -fold increase) ( $0.10<P<0.48$  and  $0.08<P<0.49$ , respectively, compared to values before challenge, depending on day). Proportion of neutrophils and lymphocytes were outside the reference range 24 h after inoculation ( $0.05<P<0.06$  for both parameters).

Detailed results are in Table III.iii and reference values for haematological parameters in sheep are in Table II.v.

**Table III.iii.** Haematological findings for leucocyte (median values) in ewes with one mammary gland inoculated with *M. haemolytica* or *S. chromogens*.

Parametre	Before inoculation	After inoculation				
	D0	3 h	6 h	9 h	12 h	24 h
Total leucocytes (cells $\mu\text{L}^{-1}$ )	8,190	7,760	8,620	11,715	12,390	18,470
Neutrophils (cells $\mu\text{L}^{-1}$ )	3,236	3,939	4,212	7,326	7,918	12,346
Neutrophils (% leucocytes)	40	51	49	63	64	67
Band neutrophils (cells $\mu\text{L}^{-1}$ )	66	329	373	322	345	328
Band neutrophils (% leucocytes)	1	4	4	3	3	2
Lymphocytes (cells $\mu\text{L}^{-1}$ )	4,532	2,745	3,198	3,262	3,328	5,036
Lymphocytes (% leucocytes)	55	35	37	28	27	27
Monocytes (cells $\mu\text{L}^{-1}$ )	284	679	781	645	689	625
Monocytes (% leucocytes)	3	9	9	6	6	7
Eosinophils (cells $\mu\text{L}^{-1}$ )	72	68	56	160	110	135
Eosinophils (% leucocytes)	1	1	1	1	1	1
Basophils (cells $\mu\text{L}^{-1}$ )	0	0	0	0	0	0
Basophils (% leucocytes)	0	0	0	0	0	0

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.

Values marked with red are outside the respective reference range (Table II.v).

## Results of proteomics examinations in samples from the experimental animals

### *Findings in examinations before challenge of the experimental ewes*

Presence of cathelicidin-1 was not evident in any of the 6 milk samples from ewes before inoculation (2 samples collected from each ewe on D0, of which one from the side of the udder to be inoculated and the other from the contralateral side of the udder).

### *Findings in examinations after challenge of the experimental ewes*

Presence of cathelicidin-1 was recorded in samples from the ewe inoculated with *M. haemolytica*, specifically in 5/5 samples from the inoculated side of the udder, starting 3 h and until 24 h after inoculation. Presence of the protein was recorded in samples from both ewes inoculated with *S. chromogenes*, specifically in 9/10 samples from the inoculated side of the udder, starting 3 h and until 24 h after inoculation. Presence of the protein was not evident in any of the three pooled samples from the contralateral side of the udder of each ewe.

In milk samples from the inoculated side of the udder of all ewes, spot density of cathelicidin-1 increased, starting at the first post-inoculation sampling (3 h after inoculation) and increased further until 12 h after inoculation.

For the inoculated side of the udder, there was clear evidence that results after inoculation were significantly greater than those before inoculation ( $P=0.002$ ), but nevertheless there were no significant differences between results obtained at the various time-points when samples had been collected after challenge ( $P>0.27$ ). Further, differences between results of inoculated and non-inoculated glands were significant ( $P=0.05$ ). Details are in Table III.iv and in Figures III.3 and III.4.

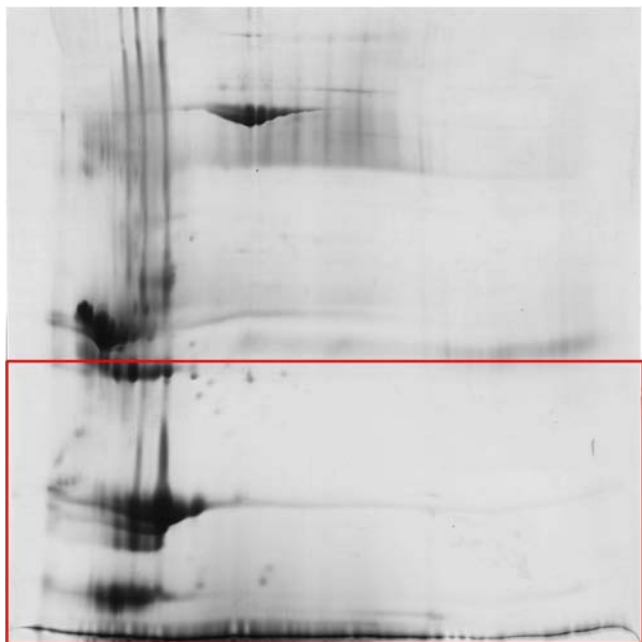
**Table III.iv.** Spot densities (mean±standard error of the mean) of cathelicidin-1 in 2-DE gels from sequential milk samples from inoculated or non-inoculated side of the udder, subsequently to inoculation of one mammary gland of each ewe with *M. haemolytica* or *S. chromogenes* (protein identification by MALDI-TOF MS).

Udder side	Total spots (n)	Before inoculation	Day after inoculation				
		D0	D0+12 h	D1	D2	D3	D4
Inoculated	2.6±0.7	0.0±0.0	813.1±506.7	637.4±415.6	419.1±283.1	340.4±194.3	786.6±497.4
Non-inoculated	0.8±0.4	0.0±0.0	0.0±0.0	68.2±60.4	14.8±14.8	2.8±2.9	0.0±0.0



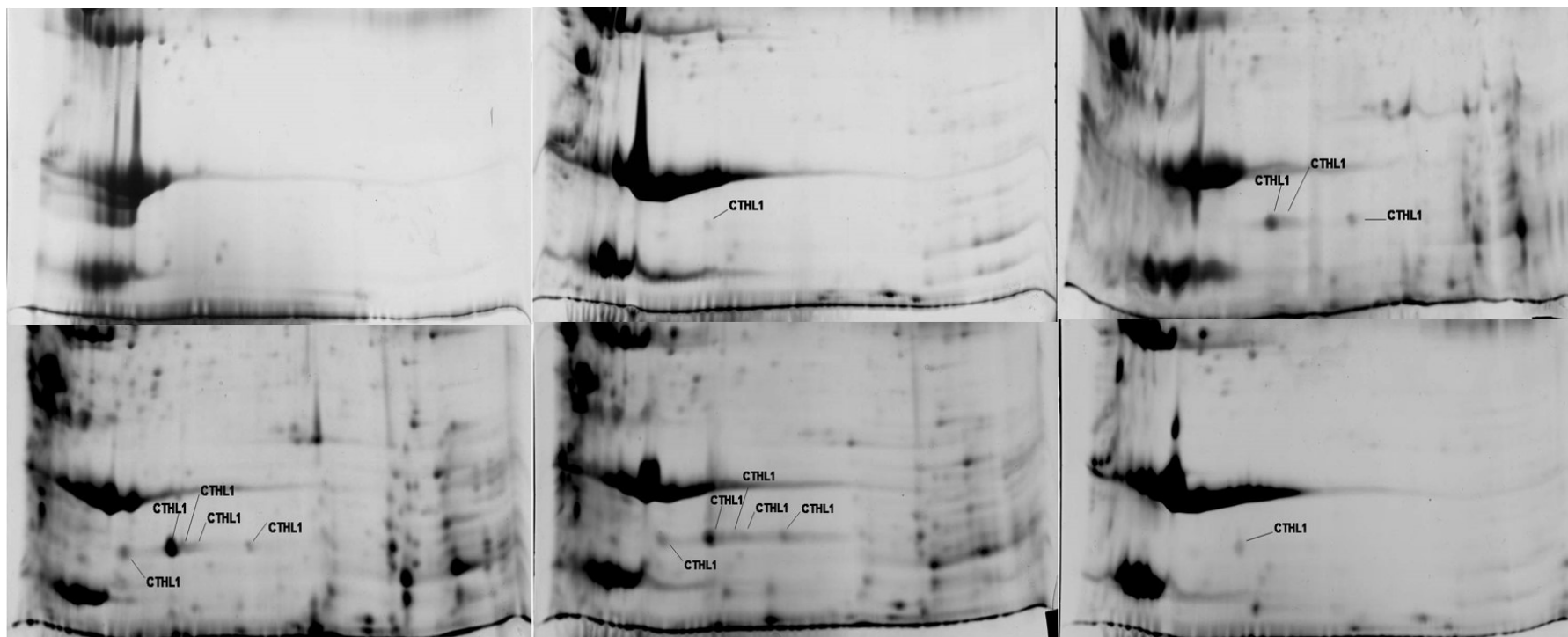
**Figure III.3.** 2-DE gels with annotation of cathelicidin-1, obtained from milk samples collected from the inoculated side of the udder of a ewe before or after inoculation of the ipsilateral gland with *S. chromogenes* or from the non-inoculated side of the udder (protein identification by MALDI-TOF MS).

(a) 2-DE gel obtained from milk sample collected before challenge from the inoculated side of the udder of a ewe; area characterised in red indicates the region of the gels shown in detail in (b) and (c).

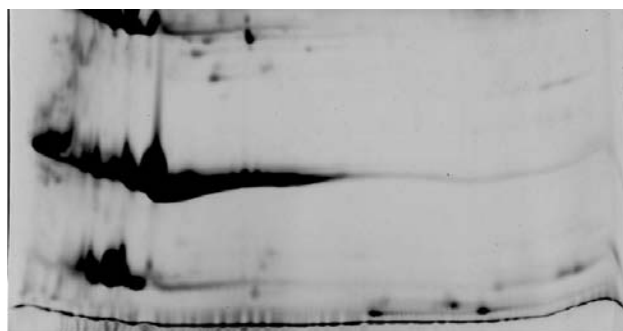


Horizontal axis: isoelectric point 3 to 10 (non-linear) from left to right; vertical axis: molecular weight 10 to 100 kDa (non-linear) from bottom to top.

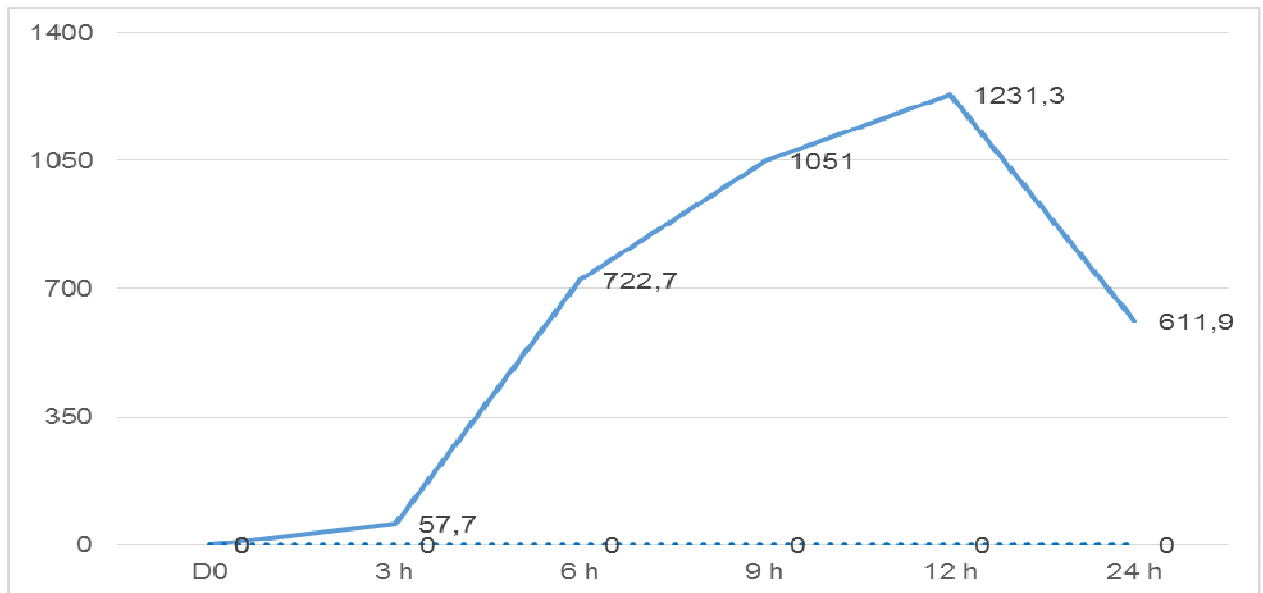
(b) Region of 2-DE gels obtained from milk samples collected sequentially before or after challenge from the inoculated gland; from top row to bottom row and from left to right: before inoculation (D0), 3 h after inoculation, 6 h after inoculation, 9 h after inoculation, 12 h after inoculation, 24 h after inoculation.



(c) Region of 2-DE gel obtained from pooled milk sample collected sequentially from the contralateral to inoculated gland.



**Figure III.4.** Mean spot densities of cathelicidin-1 in 2-DE gels obtained from sequential milk samples from inoculated (straight line) or non-inoculated (dotted line) side of the udder, subsequently to inoculation of one gland with *M. haemolytica* or *S. chromogenes*.



## D. ASSOCIATIONS BETWEEN RESULTS OF CYTOLOGICAL EXAMINATIONS AND IDENTIFICATION OF CATHELICIDIN-1 IN MILK OF EWES

### Materials and methods

#### Source of data

Detailed results of cytological examinations in the Experiment 'Proteomics analysis of blood and milk of ewes with induced *Mannheimia haemolytica* mammary infection' (Chapter II, C and Chapter III, B) (specifically: California Mastitis Test [CMT] scores) and in the Experiment 'Identification of cathelicidin-1 in milk of ewes with induced mammary infection in a blinded experiment' (Chapter III, C) (specifically: CMT scores and somatic cell counts) and results of identification of cathelicidin-1 in both Experiments have been considered for evaluation of potential associations between findings

#### Data management and statistical analysis

*Experiment 'Proteomics analysis of blood and milk of ewes with induced Mannheimia haemolytica mammary infection'*

Analysis of correlation between CMT scores and spot densities of cathelicidin-1 in milk samples was performed; for calculations, results obtained from each ewe on the respective occasion were considered. Then, a new analysis was performed, in which results were considered as 'negative' (for CMT scores: <'1', for cathelicidin-1: lack of identification) or 'positive' (for CMT scores: ≥'1', for cathelicidin-1: identification); for this analysis  $\chi^2$  test was performed.

*Experiment 'Identification of cathelicidin-1 in milk of ewes with induced mammary infection in a blinded experiment'*

Analysis of correlation between CMT scores / somatic cell counts and spot densities of cathelicidin-1 in milk samples was performed. For calculations, results obtained from each ewe on the respective occasion were considered. Then, a new analysis of correlation was performed,

where results were considered as 'negative' (for CMT scores: <'1', for somatic cell counts:  $<0.5 \times 10^6$  cells mL<sup>-1</sup>, for cathelicidin-1: lack of identification), 'doubtful' (only for somatic cell counts:  $\geq 0.5 \times 10^6$  cells mL<sup>-1</sup> and  $<1.0 \times 10^6$  cells mL<sup>-1</sup>) or 'positive' (for CMT scores:  $\geq$ '1', for somatic cell counts:  $\geq 1.0 \times 10^6$  cells mL<sup>-1</sup>, for cathelicidin-1: identification); for this analysis  $\chi^2$  test and Fisher exact test were performed.

#### *Sensitivity / Specificity of detecting cathelicidin-1 for diagnosis of mastitis*

For calculation of the sensitivity / specificity of detecting cathelicidin-1 for diagnosis of mastitis, the total number of milk samples examined during the first or the second experiment (n=92) was taken into account. Mastitis included clinical or subclinical mastitis. Clinical mastitis was defined as presence of clinically evident changes in a mammary gland (including the mammary secretion). Subclinical mastitis was defined as moderately increased somatic cell counts ( $>0.5 \times 10^6$  cells mL<sup>-1</sup>) or 'positive' ( $\geq$ '1') CMT score milk with simultaneous bacterial isolation from the sample.

Sensitivity of the test was defined as the probability that the test correctly indicated 'disease' among samples collected from mammary glands with mastitis. Specificity of the test was defined as the probability that the test correctly did not indicate 'disease' among samples collected from mammary glands with mastitis.

In all cases, an electronic data analysis tool was employed (Vassar Stats: Website for Statistical Computation; Lowry 2012, 2015). Significance level was set at  $P \leq 0.05$ .

## **Results**

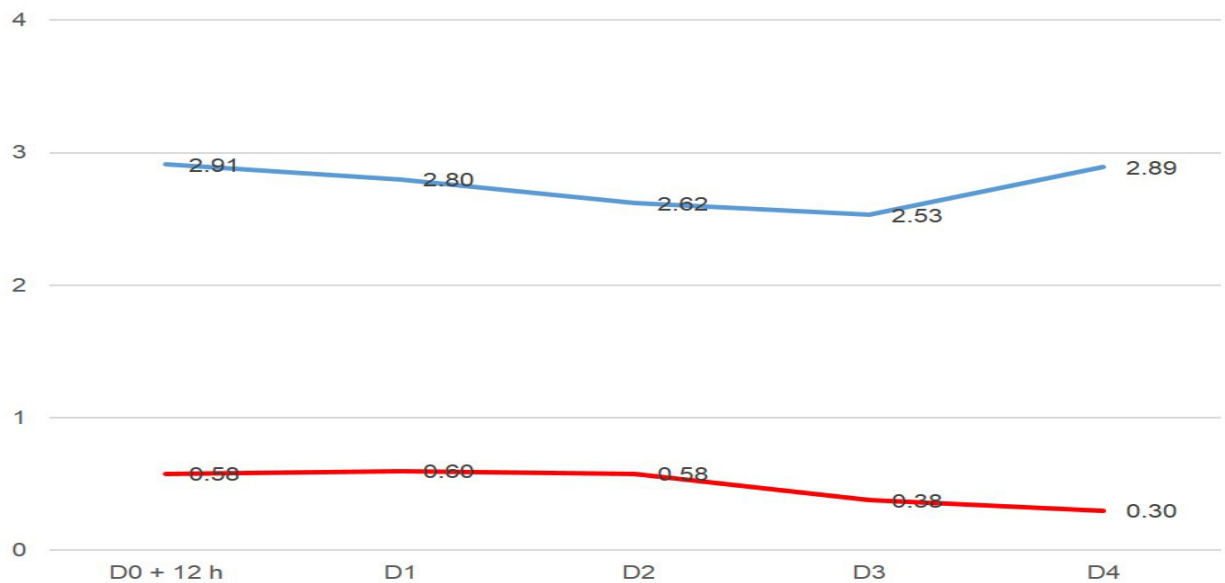
**Correlation between results of California Mastitis Test scores and cathelicidin-1 spot densities in milk samples from the experimental animals in the Experiment 'Proteomics analysis of blood and milk of ewes with induced *Mannheimia haemolytica* mammary infection'**

After inoculation, cathelicidin-1 was identified in milk samples at the same sampling as the increased CMT scores, i.e., in samples collected 12 h after inoculation.

There was significant correlation between CMT scores and cathelicidin-1 spot densities in milk samples (n=56). Specifically, correlation coefficient was  $r=0.609$  ( $P<0.001$ ). When results were considered as 'negative' or 'positive', there was a significant correlation between CMT scores

and cathelicidin-1 spot densities ( $P<0.001$  for all comparisons). Details are in Figure III.5 and Table III.v.

**Figure III.5.** Mean logarithmic CMT scores (red line) in sequential milk samples and mean logarithmic spot densities of cathelicidin-1 in 2-DE gels (blue line) obtained from the same samples collected subsequently to inoculation of the udder with *M. haemolytica*.



**Table III.v.** 2x2 contingency table indicating number of milk samples with CMT scores ('positive' [+] or 'negative' [-]) in relation to identification of cathelicidin-1 ('positive' [+] or 'negative' [-]).

		CMT score	
		<div>                     + (n=20) </div>	<div>                     - (n=36) </div>
Cathelicidin-1 spot density	<div>                     + (n=24) </div>	18	6
	<div>                     - (n=32) </div>	2	30

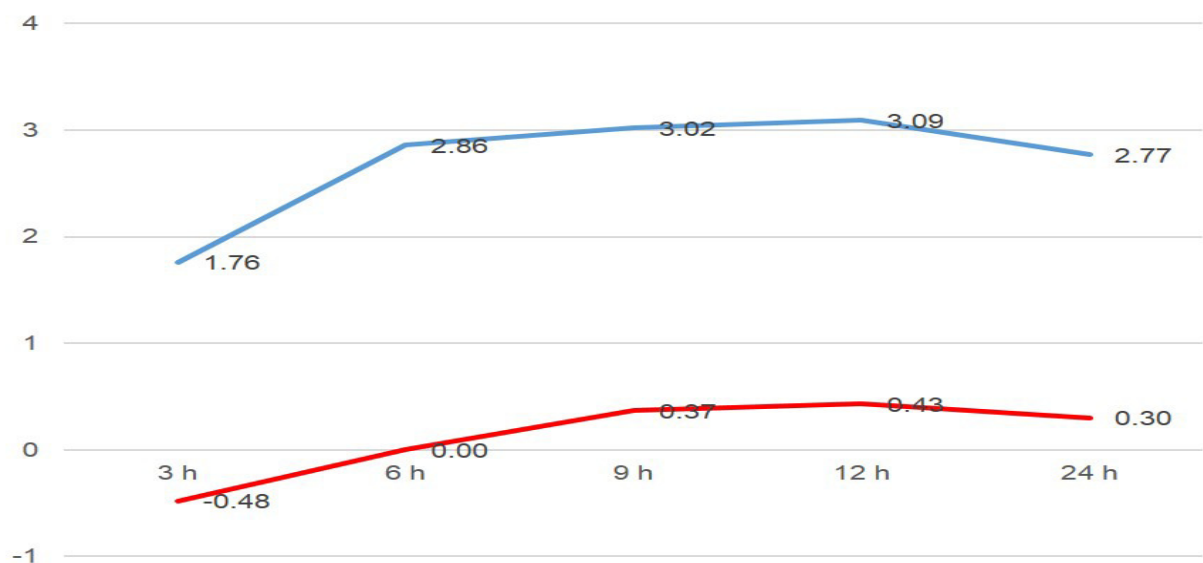
Correlation between results of California Mastitis Test scores / somatic cell counts and cathelicidin-1 spot densities in milk samples from the experimental animals in the Experiment 'Identification of cathelicidin-1 in milk of ewes with induced mammary infection in a blinded experiment'

After inoculation, cathelicidin-1 was identified in milk samples sooner than the increased CMT scores or somatic cell counts. Specifically, cathelicidin-1 was identified in samples collected 3

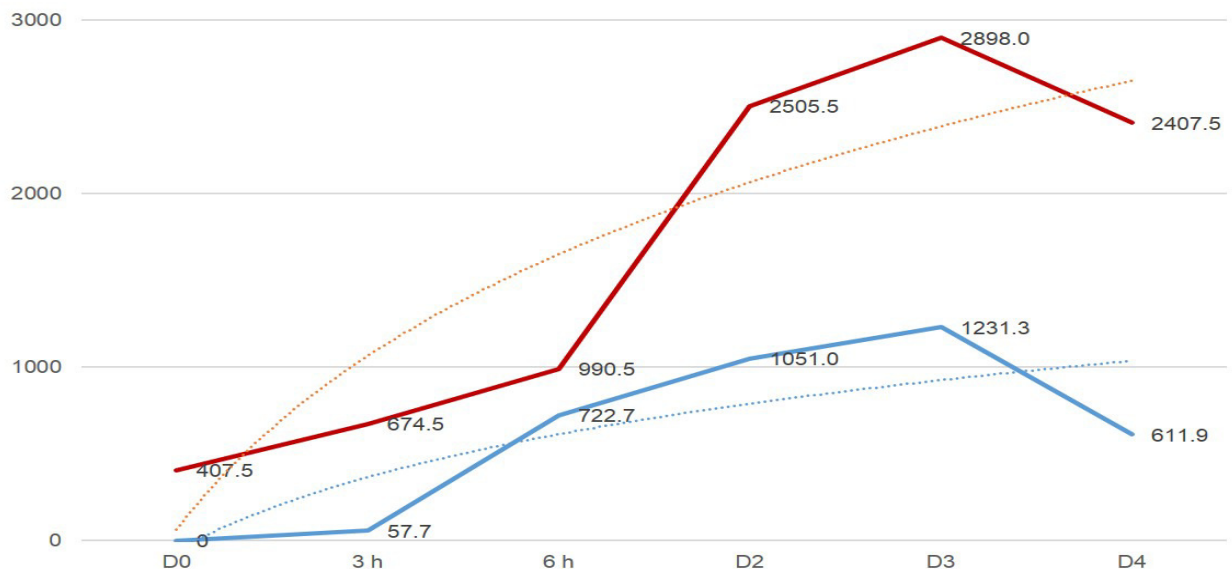
h after inoculation; in contrast, CMT scores became positive and somatic cell counts increased to values  $>1.0 \times 10^6$  cells mL<sup>-1</sup> in the sample collected from one ewe 6 h after inoculation and in the samples collected from the two other ewes 9 h after inoculation.

There was significant correlation between CMT scores and cathelicidin-1 spot densities, and between somatic cell counts and cathelicidin-1 spot densities in samples from inoculated or non-inoculated glands (n=36). Specifically, correlation coefficients were  $r=0.281$  and  $r=0.524$ , respectively ( $P=0.048$  and  $P<0.001$ , respectively). When results were considered as 'negative', 'doubtful' or 'positive', there was a significant correlation between CMT scores and cathelicidin-1 spot densities, as well as between somatic cell counts and cathelicidin-1 spot densities ( $P<0.001$  for all comparisons). Details are in in Figures III.6 and III.7. and Tables III.vi. and III.vii.

**Figure III.6.** Mean logarithmic CMT scores (red line) in sequential milk samples and mean logarithmic spot densities of cathelicidin-1 in 2-DE gels (blue line) obtained from the same samples subsequently to inoculation of a mammary gland with *M. haemolytica* or *S. chromogenes*.



**Figure III.7.** Mean somatic cell counts ( $\times 10^6$  cells mL<sup>-1</sup>) (red lines) in sequential milk samples and mean spot densities of cathelicidin-1 in 2-DE gels (blue lines) obtained from the same samples before and subsequently to inoculation of a mammary gland with *M. haemolytica* or *S. chromogenes* (logarithmic trend lines in dots).



**Table III.vi.** 2×2 contingency table indicating number of milk samples with CMT scores ('positive' [+] or 'negative' [-]) in relation to identification of cathelicidin-1 ('positive' [+] or 'negative' [-]).

		CMT score	
		<div>+</div> <div>(n=10)</div>	<div>-</div> <div>(n=26)</div>
Cathelicidin-1 spot density	<div>+</div> <div>(n=14)</div>	10	4
	<div>-</div> <div>(n=22)</div>	0	22

**Table III.vii.** 2×3 contingency table indicating number of milk samples with somatic cell counts ('positive' [+], doubtful [±] or 'negative' [-]) in relation to identification of cathelicidin-1 ('positive' [+] or 'negative' [-]) in the samples.

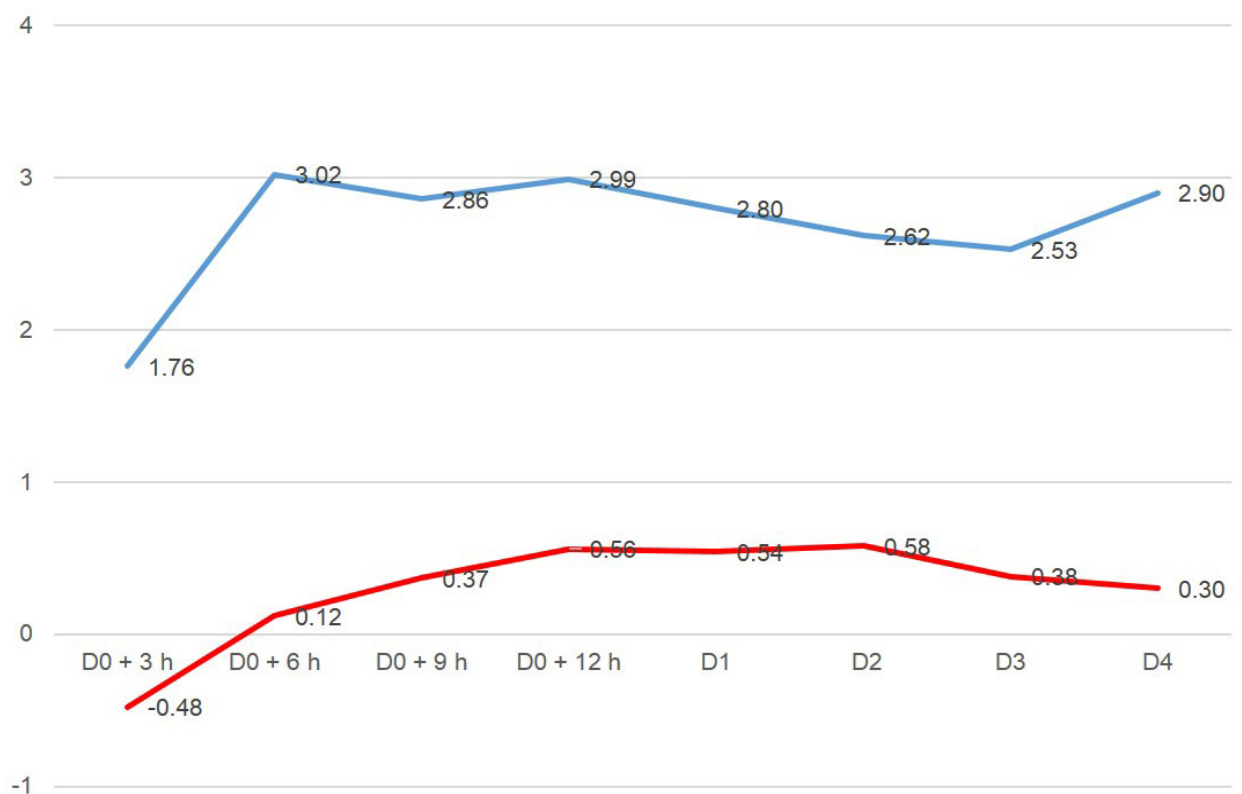
		Somatic cell counts		
		<div>+</div> <div>(n=10)</div>	<div>±</div> <div>(n=5)</div>	<div>-</div> <div>(n=21)</div>
Cathelicidin-1 spot density	<div>+</div> <div>(n=14)</div>	10	4	0
	<div>-</div> <div>(n=22)</div>	0	1	21



## Correlation between results of California Mastitis Test scores and cathelicidin-1 spot densities in milk samples from the experimental animals in both experiments

There was significant correlation between CMT scores and cathelicidin-1 spot densities in all milk samples ( $n=92$ ). Specifically, correlation coefficient was  $r=0.585$  ( $P<0.001$ ). When results were considered as 'negative' or 'positive', there was also a significant correlation between CMT scores and cathelicidin-1 spot densities ( $P<0.001$  for all comparisons). Details are in Figure III.8 and Table III.viii.

**Figure III.8.** Mean logarithmic CMT scores (red line) in sequential milk samples and mean logarithmic spot densities of cathelicidin-1 in 2-DE gels (blue line) obtained from the same samples subsequently to inoculation of a mammary gland with *M. haemolytica* (two experiments) or *S. chromogenes* (one experiment).



**Table III.viii.** 2×2 contingency table indicating number of milk samples with CMT scores ('positive' [+] or 'negative' [-]) in relation to identification of cathelicidin-1 ('positive' [+] or 'negative' [-]).

		CMT score	
		+ (n=30)	- (n=62)
Cathelicidin-1 spot density	+ (n=38)	28	10
	- (n=54)	2	52

### Sensitivity / Specificity of detecting cathelicidin-1 for diagnosis of mastitis in milk samples from the experimental animals in both experiments

Sensitivity / Specificity of using detection of cathelicidin-1 for diagnosis of mastitis was 0.965 (95% confidence intervals: 0.810-1.000) (26/27) / 0.815 (0.700-0.900) (53/65), respectively. Positive predictive value / Negative predictive value was 0.685 (0.565-0.785) / 0.980 (0.885-0.995). Details are in Table III.ix.

**Table III.ix.** 2×2 contingency table indicating number of milk samples from mammary glands with mastitis ('positive' [+] or 'negative' [-]) in relation to identification of cathelicidin-1 ('positive' [+] or 'negative' [-]).

		Case of mastitis	
		+ (n=27)	- (n=65)
Cathelicidin-1 spot density	+ (n=38)	26	12
	- (n=54)	1	53

## **GENERAL DISCUSSION**

## Introduction

Proteomics is the large-scale study of protein expression, protein-protein interactions or post-translational modifications (Gingras et al. 2007, Witze et al. 2007). Unlike other methodologies that analyse only a few proteins at a time, proteomics can provide information about many proteins in a single experiment. This ability to provide information about the entirety of proteins gives the field of proteomics the unique capability to demonstrate how cells can dynamically respond to changes in their environment. Therefore, a general goal of proteomics is to identify new and potentially unexpected changes in protein expression (which indicates gene expression), interaction or modification as the result of an experimental treatment.

Generation of large proteomic data sets is expected to demonstrate the interdependence of cellular processes important for normal cell growth or the cellular response to diseases or other abnormal conditions. In essence, a proteomic approach enables an investigator to view the whole picture of cellular functions instead of the action of one protein. This type of research enables discovery of unexpected connections between cellular processes as a precursor to new hypotheses.

Proteomics in animal sciences can be used to survey proteins expressed in cells, cell compartments, tissues or fluids (Lippolis and Reinhardt 2005, Radosevich et al. 2007). Some of the results can lead to hypothesis-driven research. Proteins, pathways or both that are modified by experimental treatments (Boyce et al. 2006, Lippolis et al. 2006) provide information for future research. These approaches can be used to examine host-pathogen interactions and to identify key bacterial or host proteins important to disease progression and recovery. Proteins significantly altered in their expression or location or post-translationally modified in animals with disease compared to healthy individuals, represent protein targets for diagnostic tests, new therapies or biomarker discovery.

The general objective of the present thesis was to increase scientific knowledge in ovine mastitis, a significant disease of ewes, by application of proteomics methodologies. In Greece, application of proteomics methodologies in animal or veterinary science has started only recently, as part of a large EU funded project entitled 'Application of "omic" technologies for the genetic characterization of indigenous Greek dairy breeds of goats and sheep, for the improvement of their production and for the identification of potential bioactivity of their milk' (short title: GOSHOMICS), which had taken place from 2012 to 2015 and had been coordinated by the Department of Obstetrics and Reproduction of the Veterinary Faculty of the University of Thessaly. That project included the first ever proteomics work performed in Greece in animal or veterinary

science, which, specifically, had been performed at the Proteomics Research Unit of the Biomedical Research Foundation of the Academy of Athens. As part of that work detailed proteomics investigations of blood and milk of healthy sheep had been performed.

## **Proteomics findings after deposition of *Mannheimia haemolytica* into the teat duct of ewes**

### **General consideration**

The study has revealed blood and milk proteome changes that had occurred during the acute phase of mastitis in sheep. A challenge model of depositing the inoculum into the teat duct of ewes, rather than inoculating the bacterial strain directly into the gland cistern as previous researchers had performed in relevant experiments (El-Masannat et al. 1991), has been used. This approach has resembled closely to natural infections and has provided more accurate information regarding the stages and the outcome of the infection (Mavrogianni et al. 2005, 2006). Further, this challenge model is particularly appropriate in the study of *Mannheimia haemolytica*, which is transmitted from the tonsils and nasopharynx of lambs into the teat duct of their dams (Fragkou et al. 2011).

Development and establishment of mastitis has been confirmed by standard methods that have for long been considered as the appropriate criteria (Fthenakis and Jones 1990): development of local clinical signs for confirmation of clinical mastitis or, in case of subclinical disease, results of bacteriological (bacterial isolation) and cytological (increased cellular content) examination of milk. Results of haematological examination further indicated development of inflammation, as evidenced by the increased neutrophil or immature neutrophil numbers. Ultimately, all animals showed histopathological findings of similar severity, as indicated by similar lesion scores marked during the histological examination. Based on above, it has been established that animals developed mastitis.

This is the first proteomics study of *M. haemolytica*-associated mastitis. Full proteomic maps have been constructed in a blood sample from one ewe collected before inoculation, in two milk samples from two ewes also collected before inoculation (one of these was the animal, for which the proteomic map in blood had been produced) and in one milk sample collected from one ewe 12 hours after inoculation. The decision to produce proteomic maps was based on the abundance of proteins on the respective two-dimensional electrophoresis (2-DE) gels, which had

subsequently supported identification of increased numbers of proteins. In any case, it should always be considered that lack of complete details for sheep genome sequencing has led to reduced identification rate in this work, as searches of nucleotides or peptide sequences might have failed to provide significant hits (Athanasiadou and Huntley 2008, Deutsch et al. 2008); this has also been reported in previous studies performed in samples from sheep (e.g., Chiaradia, Avellini et al. 2012). In fact, many of the non-identified spots led to good data, but returned no hits from the databases. In some cases, no correspondence to sheep proteins could be made. In the world database, there is no full dataset for sheep genome sequences; further, no full map of sheep proteome has become available (Mavromati et al. 2013). Nevertheless, matching could be performed to proteins of other animal species, mainly cattle, and it was considered that protein spots contained corresponding orthologs in sheep blood or milk samples.

It became evident that proteins identified in the blood sample collected before inoculation were involved in biological processes as part of the physiological role of blood: coagulation and haemostasis, transport of oxygen and ions and molecules. With reference to these results, it should be considered that, as the sample had been collected only three days post-partum, some proteins might have been increased, as a consequence of parturition, which had taken place only a short time earlier; such an example is serum amyloid A, which had been identified with increased values in blood in post-parturient cows (Alsemgeest et al. 1993). Further, proteins identified in the milk samples before inoculation were also involved in biological processes during lactation and participated in metabolic role of the mammary gland. The results indicated that only some proteins were common in both blood and milk. This is due to the limited communication between blood and milk in healthy animals. In the mammary gland, tight junctions between the epithelial cells form an effective seal surrounding each mammary epithelial cell at the apical border, acting to regulating influx of molecules through the paracellular pathway (Nguyen and Neville 1998). These tight junctions of the alveolar cells are impermeable during lactation, although various physiological factors (e.g., oxytocin, milk accumulation into the alveolar lumen) may influence their permeability leading to movements of molecules (Nguyen and Neville 1998). The higher number of proteins identified in milk compared to blood reflects the defensive abilities of the mammary gland, which is perennially exposed to infections throughout a lactation period and has limited communication with blood.

Moreover, the results have indicated that already 12 hours subsequently to bacterial deposition into the teat, new expression and/or upregulation of increased number of proteins was evident, primarily in milk and to a lesser degree in blood. In the latter tissue, the phenomenon was also evident later in the course of the study (delayed expressions). In both blood and milk, most

newly expressed or upregulated proteins were found to cluster into biological processes related to roles in inflammatory or defence response. Some of these proteins were found to change status in both blood and milk, e.g., haptoglobin or alpha-1-antiproteinase. Others have been seen only in one of these tissues; for example cathelicidin-1 has been identified only in milk, whilst complement proteins have been identified only in blood.

Interactions between blood and milk have been clearly evident. Serum albumin has downregulated in blood one day after inoculation, whilst it upregulated in milk already 12 h after bacterial deposition. There is rich documentation regarding influx of blood constituents, e.g., leucocytes, into the milk. Indeed, during mammary infection, there is increased permeability of the blood-milk barrier, which occurs by means of various mechanisms, e.g., by modulating claudins at the tight mammary junctions (Kobayashi et al. 2013) and facilitates entrance of blood constituents and molecules into milk.

The reverse phenomenon can also occur. Molecules and, more importantly, pathogens may pass from the mammary gland into the blood circulation. The salient effects of this phenomenon are bacteraemia (in case of bacterial entrance from mammary gland into blood) or endotoxaemia (in case of entrance of bacterial toxins, e.g., of *Escherichia coli*, into blood).

## Significance of proteins with differential expression after *M. haemolytica* inoculation

### *Proteins differentially expressed in blood*

#### Proteins involved in inflammatory and defence response

Alpha-1-antiproteinase, a serine and protease inhibitor, prevents, reduces or ceases activity of serine-type endopeptidases, which are enzymes catalysing hydrolysis of non-terminal peptide bonds in a polypeptide chain; endopeptidases have a serine residue (and a histidine residue) at their active centre (Kalsheker 1989). Bacterial pathogens, including *M. haemolytica* (Singh et al. 2011), produce an array of proteases acting as toxin factors, which support and enhance evasion of host defenses, facilitation of dissemination, tissue colonisation and, finally, tissue damage (Turk, Piras et al. 2012). Protease inhibitors have been considered to be immunoregulatory with anti-inflammatory effects (Safavi and Rostami 2012). Thus, downregulation of this protein in blood (as well as in milk) at two to three days after challenge coincides with subsidence of the infection, as documented by the reduced isolation rate of the challenge organism from milk samples.

Alpha 2-macroglobulin is a broad-spectrum inhibitor of serum proteases and bacterial proteases and, further, it also been characterised as an acute phase protein in various pathological conditions (Bohe et al. 1986, Banks et al. 1991, Jinbo et al. 2002). The upregulation of the protein in the present study, for a short period after infection, confirms the above roles.

Complement C3 participates in the complement cascade, in fatty acid metabolism, in immunity and inflammatory response and in lipid metabolism. Complement C3 and complement factor B are directly involved in activation of the complement cascade, which represents a first line response of the innate immune system to infection caused by various pathogens. During mammary infection, the complement system plays a role in the host's defences against invading bacteria through its bactericidal and opsonising properties; specifically, the alternative complement pathway is activated, with deposition of C3b and C3bi on bacteria and generation of C5a (Rainard 2003). Pathogens opsonised by IgG or complement have increased affinity to phagocyte Fc or complement C3b receptors, respectively, promoting phagocyte-bacteria attachment and bacterial phagocytosis (Fragkou et al. 2017).

Fibrinogen provides monomers, under the action of thrombin; these, together with fibrinogen alpha and fibrinogen gamma, polymerise to create an insoluble fibrin matrix. Fibrin plays a particular and significant role in haemostasis and participates in the initial stages of wound repairing for stabilising the lesion and guiding cell migration during restructuring of vascular endothelium. The protein participates in thrombocyte aggregation, although it is not anymore considered to be paramount in formation of thrombus under in vivo conditions (Ni et al. 2000). Hence, possibly, fibrinogen gamma-B chain upregulation recorded in this study can be associated with *Mannheimia*'s leucotoxin damage to thrombocytes, as discussed above, as well as with effects of the pathogen in causing vascular damage and micro-haemorrhages in mammary vessels (El-Masannat et al. 1991). Further, fibrinogen beta chain was identified with downregulation in blood and upregulation in milk soon after inoculation, indicating leakage from blood, through the altered blood-milk barrier, as discussed further below (see serum albumin).

Haptoglobin is a common acute-phase protein, which has been considered in many circumstances for diagnostic purposes (Eckersall et al. 2006). The protein mainly scavenges toxic haemoglobin released during haemolysis or normal turn-over of erythrocytes, that way regulating haemoglobin-bound iron elimination. Also, it has an antioxidant and anti-inflammatory role and is implicated in immunomodulation. During bovine mastitis, levels of alpha- and beta-haptoglobin have increased significantly in the blood of affected cows (Alonso-Fauste et al. 2012). However, Eckersall et al. (2006) have suggested that blood was unlikely to be a source of the protein for the observed increase in milk, as results have indicated that earliest time for detection of the



increased concentrations was 24 h post challenge. In the present study, haptoglobin levels were found to increase in milk 12 hours after bacterial deposition into the teat, whilst in blood three days later. Possibly, this could indicate, given also the above hypothesis by Eckersall et al. (2006), that haptoglobin might possibly be transferred from milk into blood. In this respect, one should consider that opening of the tight junctions between blood and milk does not only facilitate leakage from blood into milk, but also contributes to passage of molecules from milk into the blood circulation.

Interleucin-4 participates in the activation of various defence cells, e.g., B-lymphocytes (Lee et al. 1986). The protein induces expression of various molecules of class II major histocompatibility complex in B-lymphocytes, enhances secretion and cell surface expression of immunoglobulins G<sub>1</sub> and E (Sideras et al. 1987) and regulates expression of IL31RA by macrophages (Edukulla et al. 2015). The cytokine has been detected in cases of ovine mastitis by Rodríguez and Castro (2015), who have indicated interleucin-4 as one of the primary two cytokines (the other being tumour necrosis factor- $\alpha$ ) prevailing in the disease, both produced by mammary macrophages. Cytokines released by mammary macrophages play a role, among others, in activating B-lymphocytes (Murtaugh and Foss 2002), cells with a significant role in mammary defence (Fragkou et al. 2010). Findings of upregulation of interleucin-4 in the present study on the fourth day post-challenge are in line with mobilisation of lymphocytes during the subacute stage of the diseases.

#### Other proteins

Actin cytoplasmic 1 is a common protein involved in cell organisation (Doherty and McMahon 2008), which participates in various cellular processes, e.g., muscle contraction, cell motility, cell division and cytokinesis, establishment and maintenance of cell junctions and cell shape. In previous studies performed in cows with mastitis, the protein has been associated with the phase of cell invasion by the pathogen; specifically, in *Staphylococcus chromogenes*-associated mastitis, in which the causal bacteria directly affect mammary epithelial cells, upregulation of the protein and re-arrangement of actin cytoskeleton has been recorded (Zhang and Maddox 2000); in *E. coli*-associated mastitis, in which pathogenic action of the bacterium is exerted through its endotoxin, no such findings were evident (Passey et al. 2008). In the present study, downregulation of the protein was seen, likely because *M. haemolytica* exerts its pathogenic action through its leucotoxin, acting mainly in blood cells (leucocytes, thrombocytes, erythrocytes) (Zecchinon et al. 2005), i.e., with little direct involvement in the role of actin cytoplasmic 1. Moreover, as the protein was identified with downregulation in blood and upregulation in milk soon after inoculation, leakage from blood, through the altered blood-milk barrier, as discussed further below (see serum albumin), might have also occurred.

Antithrombin-III is also a serine and protease inhibitor, as well as regulating blood coagulation cascade (Bjork and Olson 1997). Its expression after infection may be the consequence of *M. haemolytica* action in thrombocytes of the host, which have led to increased micro-haemorrhages in affected animals.

Angiotensinogen, an essential component of the renin-angiotensin system, is a potent regulator of blood pressure, body fluid and electrolyte homeostasis (Hume et al. 2016). Angiotensinogen has been reported with downregulation in people with intestinal inflammation (Hume et al. 2016) and rats with oxidative stress and hepatic fibrosis (Reza et al. 2016). A potential association with mastitis has never been reported. Presence of this protein might be the consequence of the efforts of the infected ewes to balance milk pH, osmolality and electroconductivity, as electrolyte homeostasis is severely disturbed, given that the destruction of the mammary epithelial cells opens channels of communication between blood and milk with significant modification of above parameters in ewes with mastitis (Fragkou 2009).

Apolipoprotein A-I is involved in phospholipid and reverse cholesterol transport. Also, it functions as a cofactor for lecithin-cholesterol acyl transferase and possesses anti-inflammatory and anticoagulant properties (Jonas 1975). It has also been suggested that the protein inhibited A-beta peptide aggregation and toxicity (Liao et al. 2005, Yan et al. 2006). The protein has also been described as a 'negative' acute-phase protein (Oikawa et al. 1997, Oikawa and Katoh 2002, Khovidhunkit et al. 2004) (i.e., a protein with decreasing concentration in inflammation, in order to save amino acids for positive acute phase proteins - Abbas et al. 2012), although other researchers (Alonso-Fauste et al. 2012) have not agreed with that hypothesis, a view which seems to be corroborated by present results: upregulation of apolipoprotein A-I was evident in milk 12 hours and in blood three days after bacterial deposition into the teat. Although the protein is considered to be involved primarily in metabolic processes (lipid metabolism, cholesterol metabolism, steroid metabolism) (Khovidhunkit et al. 2004), it may also play a defensive role participating in complement activation (Turk, Kovacic et al. 2012, Turk, Piras et al. 2012) and, possibly, in inhibition of bacterial proteases (Turk, Piras et al. 2012). These roles would likely be fulfilled during its upregulation.

Apolipoprotein A-IV, a high-density lipoprotein, is atheroprotective, with properties and role similar to those of apolipoprotein A-I as described above. Khovidhunkit et al. (2004) have indicated apolipoprotein A-IV as an acute-phase protein present in blood circulation. Present results are in line with that assumption, as the protein showed increase shortly after inoculation and for only a short period.

Beta-2-glycoprotein 1 (or apolipoprotein H) binds to various kinds of negatively charged substances, e.g., heparin, phospholipids, dextran sulfate. It may prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells (Miyakis et al. 2004) and has a complex involvement in agglutination, as it appears to alter adenosine diphosphate mediated agglutination of thrombocytes (Nimpf et al. 1985). Usually, it assumes an anti-coagulation activity in serum (by inhibiting coagulation factors), however changes in blood factors can result of a reversal of that activity (Miyakis et al. 2004). Likely, in the present study, it has been identified, because it might participate somehow (but still unclearly) in the host reaction to destruction of thrombocytes by *M. haemolytica* leucotoxin (Zecchinon et al. 2005).

Ceruloplasmin is a copper-binding glycoprotein, involved in iron transport across cell membrane; it also oxidises iron, facilitating for ferritin and inhibiting microbe iron uptake (Tabrizi et al. 2008), a function of particular significance in this model, given the demand of *M. haemolytica* for iron (Al-Sultan and Aitken 1984). It is considered as an acute-phase protein, although in the present study it showed a fluctuating pattern after inoculation, findings similar, to a large extent, to those of Tabrizi et al. (2008), who have reported no changes in ceruloplasmin in blood samples from cows with mastitis.

Cysteine and histidine-rich domain-containing protein 1 has been proposed to acting as co-chaperone for heat shock protein HSP 90-alpha, hence was involved in stress response (Wu et al. 2005). In the present study, this protein was expressed in blood three days after inoculation, possibly as a result of prior presence of heat shock proteins in milk samples.

ETS-related transcription factor Elf-5 is an activator, involved in mammary gland epithelial cell differentiation. Its expression three days after challenge possibly indicates the tendency of the mammary gland for regeneration.

Gelsolin was the first member identified in a family of actin-binding proteins involved in the control of organising actin cytoskeleton in cells (Sun et al. 1999). It severs actin filament at presence of micromolar amount of calcium, thereby disassembling the actin network. Although embryonic development and longevity were normal in case of mice lacking gelsolin, migration of neutrophils and dermal fibroblasts was found to decrease (Witke et al. 1995). These findings indicate involvement of gelsolin in the leucocytic response of hosts during homeostasis, inflammatory reaction and wound healing. Further, gelsolin has been implicated in mammary development (Crowley et al. 2000), as it was required for development of the ductal part of the gland, by mediating epidermal growth factor effects on cell motility (Chen, Murphy-Ullrich et al. 1996) and subsequently development, elongation and branching of the ductal part (Hennighausen and Robinson 2001, 2005). Further, Janjanam et al. (2014) have shown increased expression of

the protein during lactation, which participated in maintenance of polarity and shape of mammary epithelial cells, thus contributing to milk secretion.

Haemoglobins and haemopexin bind and scavenge haeme released by the turnover of haeme proteins, that way preventing haeme-mediated oxidative stress and haeme-bound iron loss. Although, according to Alonso-Fauste et al. (2012), their synthesis is induced during inflammation, present findings are contrasting to that view. In the present study, levels of haemoglobins and haemopexin decreased. This may be the direct effect of *M. haemolytica* in erythrocytes, as the pathogen's leucotoxin has been reported to also cause lysis of these cells in infected sheep (Murphy et al. 1995). There is supportive evidence for this hypothesis, given that, after infection, relevant haematological parameters decreased and were occasionally below reference values. As a consequence, erythrocytes may fail to carry oxygen, leading in 'cell anaemia' (i.e., hypochromic anaemia) (Yang et al. 2009), finally resulting in respiratory burst in neutrophils (Yang et al. 2009).

Myosin-1 is a major contractile protein with participation in conversion of chemical energy into mechanical energy through adenosine triphosphate hydrolysis, which has been associated with endocytosis of immunoglobulins by cell surface binding (Yanase et al. 1997). The protein has not been associated with mastitis in previous works.

Peroxiredoxin-6 has an antioxidant role, being involved in cell defense against oxidative stress and in redox regulation of intracellular signaling (Takagi et al. 2012). It has the ability to reduce H<sub>2</sub>O<sub>2</sub> and short chain organic, fatty acid and phospholipid hydroperoxides, as well as to participate in regulation of phospholipid turnover and in protecting against oxidative injury and suppressing inflammation in nervous system (Takagi et al. 2012, Yun et al. 2015). The expression of the protein in the present study is consistent with above roles and the proteins has been reported in the past to have been associated with mastitis in cows (Alonso-Fauste et al. 2012).

Plasminogen is the precursor of plasmin, which lyses fibrin clots to various degradation products and D-dimer; the conversion to active protease is mediated by tissue-type (tPA) and urokinase-type (uPA) plasminogen activators (Mehta and Shapiro 2008). Theodorou et al. (2010) have reported upregulation of plasminogen during mastitis associated with *Streptococcus agalactiae*, work that has been followed by another one by Pisanu et al. (2015), in which similar findings in cases of mastitis associated with *Streptococcus uberis*; to note, however, that above models are of little significance for ovine mastitis, as these pathogens are rare causal organisms of the disease (Gelasakis et al. 2015). Upregulation of plasminogen has been recorded in the present study and likely is the consequence of mammary tissue remodeling occurring during the inflammatory process, when mammary alveoli and epithelial cells are destroyed by the effect of the

invading organism's virulence factors and (at a later stage) the host initiates involution of the mammary gland (Fthenakis and Jones 1990). Plasminogen fragments identified in blood can be explained as the result of action by neutrophils, which, stimulated by apolipoprotein (also identified with upregulation), contribute to generation of these fragments (Lamanuzzi et al. 2004, Smokovitis 2005).

Retinol-binding protein 4 delivers retinol from the liver stores to peripheral tissues. In plasma, the retinol-binding protein-retinol complex interacts with transthyretin, that way preventing losses through renal glomeruli. The protein has been reported to be involved in immunity and defensive mechanisms (Moraes-Vieira et al. 2014) and in inflammatory process (Farjo et al. 2012) and has been described as a 'negative' acute phase protein (Güdücü et al. 2014). Present findings are not in accord with those, however, as the protein has been found to increase after infection.

Serotransferrin is involved in transport and distribution of iron and in degradation of haeme for storage and utilisation. Further, it may have a role in stimulation of cell proliferation (Crichton and Charleaux-Wauters 1987) and has also been described as a 'negative' acute phase protein (Kushner 1982). Alonso-Fauste et al. (2012) have reported a decrease of the protein in blood during bovine mastitis. The present results would query this view, given that, although there was a marked decrease in blood immediately after challenge, an upregulation was evident in milk 12 hours after inoculation, which indicates that the protein leaked from blood into milk, as discussed below (see serum albumin).

(Serum) Albumin is the most abundant protein in blood and fulfils multiple functions, transporting various molecules, regulating oncotic pressure (colloid osmotic pressure) and playing a principal antioxidant role (Dinarello 1999). In cases of bovine mastitis, serum albumin has been found to decrease in blood of affected animals and also its isoelectric focusing pattern has changed (Alonso-Fauste et al. 2012). In the present study, serum albumin was found reduced in blood and increased in milk soon after challenge. Those findings are compatible with the model of increased permeability of 'blood-milk' barrier, as the result of which blood proteins and leucocytes enter into infected mammary glands. Although only one study in goats has shown no increased presence of blood serum albumin in the milk (Olumee-Shabon et al. 2013), suggesting no breakdown of the barrier in such circumstances, the findings indicate clearly that, during mastitis, blood constituents leak into the milk and, hence, that the prevailing model is valid.

Transthyretin is involved in transport of thyroid hormones and retinol, a role that is affected by inflammation and malnutrition (Myron Johnson et al. 2007). It is also important as inhibitor of interleucine-1 production by monocytes and endothelial cells, and thus plays an anti-inflammatory role (Borish et al. 1992), described as a 'negative' acute phase protein. Changes in status of this

protein during inflammation have been considered as the result of the change in small molecules binding to endocrine proteins (Bernstein 2009). There are conflicting reports regarding status changes of transthyretin in the blood of cows with mastitis; Rezamand et al. (2007) reported delayed decrease of blood concentrations of transthyretin, whilst, in contrast, Turk, Piras et al. (2012) have indicated increased ones. In the present study, a pattern similar to that of serum albumin has been recorded: decrease in blood with concurrent upregulation in milk, consistent with a leakage from blood to milk.

Tuftelin-interacting protein 11 is a splicing factor that has been identified as a component of the nuclear spliceosome (Zhou et al. 2002); the spliceosome is a large complex composed of small nuclear ribonucleoproteins (snRNPs) and non-snRNP associated proteins, functioning together to mediate removal of introns from pre-mRNAs. The protein is localised to novel subnuclear speckles being in close proximity to, but distinct from, nuclear speckled regions characteristic of many splicing factors or snRNPs (Wen et al. 2005). Tuftelin-interacting protein 11 is involved in maintaining efficient intron removal by accelerating the recycling of functional snRNPs (Tannukit et al. 2009). In this study, a pattern similar to that of serum albumin has been recorded: decrease in blood with concurrent upregulation in milk, consistent with a leakage from blood to milk.

Vitamin D-binding protein is involved in transport and storage of that vitamin, in scavenging of extracellular G-actin, in enhancing chemotactic activity of complement 5 $\alpha$  for neutrophils during inflammation and in activation of macrophages (Nagasawa et al. 2005). Results of the present study have indicated upregulation or new expression of the protein in blood or in milk, respectively, findings similar to those reported in cows with mastitis (Boehmer 2011, Turk, Piras et al. 2012), as it contributes to macrophage activation (Seth et al. 2009), which is the first line of cellular mammary defences.

#### *Proteins differentially expressed in milk*

As discussed above, the identification of actin, cytoplasmic 1, fibrinogen beta chain, serotransferrin, serum albumin, transthyretin and tuftelin-interacting protein 11 with downregulation in blood and upregulation in milk soon after challenge indicates leakage of blood constituents from blood into milk. Changes in permeability of the blood-milk barrier in cases of infection have been long established and well documented (Schalm et al. 1971, Craven and Williams 1985). These take place under the action of virulence factors released from invading organisms, as well as of cytokines produced by mammary macrophages (Boehmer et al. 2008); they serve, primarily, to achieving influx of leucocytes (initially neutrophils) into the infected mammary gland (Cranen and

Williams 1985), a main defence mechanism occurring in all cases of tissue injury (Hurley 1983). As the result of these changes, proteins also leak into the milk and, in taking a teleological approach to that, they are fulfilling precise roles in the affected mammary gland; for example, actin, cytoplasmic  $\alpha$  participates in re-organisation of cells of affected mammary parenchyma, fibrinogen beta chain contributes in repair of injured tissue, stabilising lesions and restructuring vascular endothelium, serotransferrin partakes in ion transport in the mammary gland, as extensive ion ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ) changes take place on such cases, and serum albumin supports equilibration of altered osmolality (by means of the oncotic pressure which it regulates) in milk.

In this hypothesis, protein influx into the affected mammary gland is not an event taking place solely because permeability of the blood-milk barrier has been modified, but has an active role contributing in efficient mammary defence, as well as in repair of tissue damage and proper refuction of the mammary gland. It is noteworthy that some of these proteins (serotransferrin, serum albumin, tuftelin-interacting protein 11) have also been identified with upregulation in milk of the contralateral gland. It is also interesting that proteins with clear defensive role, e.g., cathelicidin-1, have also been identified in milk of the contralateral gland. The findings indicate, first of all, that changes in permeability of the blood-milk barrier are likely regulated by the host's response, as, of course, bacterial virulence factors prevail mostly in the affected mammary gland. Further, presence of neutrophil-associated proteins (cathelicidins) indicates neutrophil influx into the non-infected gland, although, these cells are difficult to detect, because increased cell numbers have rarely detected in contralateral to infected glands (Fthenakis and Jones 1990, Mavrogianni et al. 2005, Fragkou et al. 2010) and as also evidenced by cytological results of the presence study, although in a study carried out in female dogs (Ververidis et al. 2007) increased cellular content in mammary glands other than the infected one has been reported.

One may thus postulate, with the hypothesis supported by the results, that teleology of protein and cell infiltration into the contralateral mammary gland is its preparedness to counteract potential bacterial invaders. This is not unreasonable; portal of entry of bacteria into the mammary gland is the teat and, most often, both teats of an animal are exposed to similar environmental stimuli and challenges. It is well documented that, in most cases of mastitis in ewes (>90%), only one mammary gland is affected (Fthenakis 1994, Gelasakis et al. 2015). It may thus be possible that the 'preparation' of the host as discussed above, by presence of cells (for defence) and proteins (for defence and wound repair) effectively counteracts and limits potential bacterial infections taking place at the contralateral to the initially infected mammary gland, as well as leading to healing of ensued damages.

### Proteins involved in inflammatory and defence response

Reasonably, many proteins differentially expressed in milk after inoculation (in total 13) were involved in the inflammatory and defence response of the experimental ewes. Specifically, these were related to acute phase response (e.g., haptoglobin), to antibacterial activity (e.g., cathelicidin-1) or to immune response (e.g., beta-2-microglobulin).

Addis et al. (2013) have also reported identification of proteins upregulating after mammary infection, which were involved in innate immune response processes, e.g., lactotransferrin, cathelicidins, calprotectin subunit S100-A9, complement C3, haptoglobin. In other studies, results have indicated that various families of antimicrobial proteins had upregulated in the mammary tissue of cows with mastitis; among these, members of the cathelicidin family of proteins (cathelicidin 1, 2, 4) (Zanetti et al. 1995, Tomasinsig et al. 2010, 2012, Smolenski et al. 2011).

Beta-2-microglobulin is part of the major histocompatibility complex class I molecules (Güssow et al. 1987) and is involved in the presentation of peptide antigens to the immune system. Lack of identification of this protein in previous studies might be related to genetic factors. In cattle, the genetics of bovine leucocyte antigen (*BoLA*) has been linked to resistance to mastitis (Takeshima and Aida 2006). An increased prevalence of the disease was found in animals carrying the *BoLA* class II haplotype 'b' (Arriens et al. 1994), while cows carrying the *BoLA* class I CA42 allele instead of the EU28 allele had an increased susceptibility to *Staphylococcus aureus* infection (Schukken et al. 1994). It is noteworthy that in sheep, although polymorphism of major histocompatibility complex genes occurs, there is little relevant data and occasionally conflicting (Swiderek et al. 2006, Fragkou et al. 2010). Hence, genetic differences in regulation of major histocompatibility complex might possibly be reflected in expression of beta-2-microglobulin.

Cathelicidin-1 is member of a family of cationic peptides with various antimicrobial activities. Its significance has been reviewed in Chapter III, part A. Cathelicidins are synthesised as preproproteins; following secretion or neutrophil degranulation, they are cleaved by proteases (e.g., serine proteases, proteinase 3, elastase, kallikrein) to generate the cathelin domain and C-terminal antimicrobial peptides with potent activity (Boman 2003, Zanetti 2004). In fact, in milk, neutrophils release various proteins with the potential to degrade extracellular matrix proteins and lacteal proteins, as indicated by Mehrzad et al. (2005), who have reported that many of the proteases in mammary secretion during mastitis originated from milk neutrophils. Further, an increased quantity of antimicrobial proteins has been considered to be associated with reduction in abundance of caseins in cows with mastitis (Zhao, Yang et al. 2015). This is reasonable, as proteases contribute to lysis of lacteal proteins, but also support the action of cathelicidins (and



other proteins of similar function). Further, significance of identification and the potential of cathelicidin-1 in diagnosis of mastitis are discussed below (see next section of Discussion).

Haptoglobin, apart from its role in scavenging haeme, has also an antioxidant and anti-inflammatory role and is implicated in immunomodulation. It is a confirmed acute phase protein, that has been identified in milk of cows with acute mastitis (Pyörälä et al. 2011).

Lactoperoxidase is synthesised in the mammary gland, in the presence of thiocyanate of hepatic origin and hydrogen peroxide of bacterial or endogenous origins. It exerts its antibacterial activity through formation of activated oxygen products, e.g., hypothiocyanate, a metabolite enhancing bactericidal activity of leucocytes (Fragkou et al. 2017). Under *in vitro* conditions, the system has been found with significant antibacterial properties. Lactoperoxidase has been found to increase during mastitis caused by streptococci (Janota-Bassalik et al. 1977), which are not producing catalase ('catalase-negative' bacteria). In other infections, e.g., by catalase-positive *Staphylococcus aureus* (Janota-Bassalik et al. 1977) or Gram-negative *M. haemolytica* (present study), reduction after infection has been evident. Its role during the lactation period is limited, likely because of interference by other milk proteins. Possibly also, concentrations of thiocyanate ions in milk would depend on nutritional regime and small oxygen tension in the mammary gland might act as an inhibitor for production of hydrogen peroxide; these two factors may further account for the limited efficacy of the system against mastitis-causing pathogens in lactating animals (Fragkou et al. 2017).

Lactotransferrin (or lactoferrin) is an iron-binding glycoprotein, mainly produced by the secretory epithelium and in smaller quantities by neutrophils. The protein has confirmed antimicrobial (against Gram-negative bacteria) and anti-inflammatory properties (Kutilla et al. 2004) and has already been reported to increase during mastitis (Hagiwara et al. 2003, Fetherston et al. 2006), as recorded in the present study. It is an iron-binding protein, synthesised mainly by mammary epithelial cells (Harmon and Newbould 1980). Its expression in milk is inversely related to alveolar development. Possibly, its reduction soon after challenge indicates a role against the iron-demanding *M. haemolytica* (Al-Sultan and Aitken 1984), with further increase at a later stage, i.e., when infection had subsided. The antibacterial effect of lactotransferrin is expressed mainly in the epithelia lining the ducts and cisterns, but not at the proximal end of the teat canal (Fragkou et al. 2017). It is greatly enhanced in increased bicarbonate ion concentrations and reduced concentrations of the lactotransferrin inhibitor, citrate ions, which are present during the dry period. Lactotransferrin exerts a bacteriostatic effect mainly by competing with bacteria for available iron or by binding to bacterial surfaces, hence is of particular significance against Gram negative bacteria (e.g., *Mannheimia haemolytica*, a significant cause of ovine mastitis). Its function includes

the alteration of the integrity and permeability of bacterial cell wall and the sensitization of bacteria to antimicrobial agents. Also, it contributes to bacterial killing and promotes adhesion and aggregation of neutrophils to the endothelial surface, as well as being involved in the activation of the complement system via the alternative pathway and in antigen-processing by cells of the reticuloendothelial system and in antibody production (Fragkou et al. 2017).

Zhao, Yang et al. (2015) have reported upregulation of S100-A11, S100-A2, S100-A9, S100-A12 and S100-A8 in the mammary gland of cows with mastitis; these proteins belonged to the S100 multigenic family, have been identified with antimicrobial activities and bind  $\text{Ca}^{2+}$ . They are mainly produced by leucocytes (monocytes/macrophages, neutrophils) (Perera et al. 2010). S100-A12 expression could be greatly increased in mammary secretion after challenge with *E. coli* (Boehmer et al. 2008), likely as the result of mammary response to challenge (Lutzow et al. 2008). S100-A9 and S100-A8 are mainly expressed in cytoplasm of neutrophils and monocytes, as well as in activated endothelial and epithelial cells (Hu et al. 1996, Striz and Trebichavsky 2004). As a consequence, S100 proteins have been proposed as possible markers for many inflammatory diseases. Alone or in association with calprotectin (Striz and Trebichavsky 2004), S100-A8 and S100-A9 exert strong pro-inflammatory and chemotactic activities (Raquil et al. 2008) by promoting leucocyte recruitment (Ryckman et al. 2003, Vandal et al. 2003).

Serum amyloid A, an apolipoprotein, is an acute phase reactant. Varying isoforms of serum amyloid A are expressed at various levels or in response to inflammatory stimuli, considered to be produced at the liver (Uhlir and Whitehead 1999). However, present identification of this protein only in milk, but not in blood, after challenge, supports a theory presented by Eckersall et al. (2001, 2006) that the protein was also produced locally, at the mammary gland. Previously, McDonald et al. (2001) had identified an isoform of the protein present in colostrum, which they termed mammary-associated serum amyloid A. Eckersall et al. (2001, 2006) have identified this protein in mammary epithelial cells and in the gland cistern (i.e., associated with lacteal secretions), as well as, in smaller quantities (i.e., of lesser significance), in the teat duct of cows. Our findings indicate a similar pattern in ewes.

#### Other proteins

In total, nine differentially expressed proteins were involved in cell communication. These included proteins involved in actin binding (tropomyosin alpha-3 chain), ATP-binding (phosphatidylethanolamine-binding protein 1, myosin-1, myosin-7), chaperoning (T-complex protein subunit beta), cell adhesion (vinculin), positive regulation of cell migration (78 kDa glucose-regulated protein), sensory transduction (rhodopsin kinase), ubiquitin-like conjugation pathway (E3 ubiquitin-protein ligase). The chaperonins have previously been described with a role in pathogen

recognition (Triantafyllou et al. 2001), which is of significant importance in mastitis, in order to achieve effective defence by the host. Other proteins related to cell communication may be active to recruitment of leucocytes into the mammary gland and the necessity for these to circulate within the inflamed gland, in order to effectively perform phagocytosis and intracellular killing.

Further, five proteins were involved in cell death and apoptosis and all changed status 12 to 24 hours after inoculation. Caspases often enhance induction of apoptosis (programmed cell death), through a well-defined sequence of events (Wesson et al. 2000). The same authors have also found that caspase-8 (but not caspase-1, as in this study) was upregulated in bovine mammary epithelial cells after *S. aureus* infection and induced cell apoptosis (Wesson et al. 2000); caspase-1 has been considered as the target for induction of apoptosis during infections by *Salmonella* (Hersh et al. 1999) or *Shigella* (Chen, Smith et al. 1996). In any case, precise pathways of apoptotic cell death induced by caspases have not yet been fully characterised; in general, these may involve the initial stimulus, caspase activation and cleavage of protein substrates with apoptotic functions (Kidd 1998). Cathelin-related peptide SC5 belongs to the family of peptides sharing a highly conserved cathelin domain, which have antibacterial functions, as well as being associated with cytolysis and cell death (Xiao et al. 2006). Chitinase-3-like protein 1 has a role in inflammation responding to interleucine-1 and interleucine-6 (Lee, Hartl et al. 2009); it is possibly involved in the process of pathogen recognition, that way supporting initiation of the defence response. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays a role in glycolysis and nuclear functions, neuron apoptotic process, glucose metabolic process, glycolytic process, that has been considered to be associated with various disorders, including possibly with mastitis, as reported by Smolenski et al. (2007), which has also been found in the present study. Interstitial collagenase (a matrix metalloproteinase) is involved in collagen degradation and involved in tissue remodeling. In a recent study performed in milk samples from goats, an association between the protein and increased somatic cell counts has been reported (Li et al. 2016); this may be explained as a consequence of the tissue injury inflicted during bacterial mammary infection. In general, cell death-associated proteins may be indicative of the lysis of leucocytes occurring in milk as part of extensive cell death taking place during mastitis, given that in the mammary gland there is decreased oxygen availability for extended leucocyte function (Prasad and Newbould 1968, Hill 1992), as well as because, in milk, leucocytes often phagocytose casein micelles and fat globules, thus depleting their resources and facilitating earlier cell death (Reiter and Bramley 1975).

The three differentially expressed proteins involved in cell differentiation, regulated specifically angiogenesis (phosphoglycerate kinase 1), cell differentiation (glycoprotein-N-

acetylgalactosamine 3-beta-galactosyltransferase 1) or neurogenesis (nucleoside diphosphate kinase A) and were newly expressed within 24 hours after challenge.

In total, 17 differentially expressed proteins were involved in cell organisation and biogenesis. Specifically, were involved in cell shaping (ezrin), cellular response to interleucine-4 (tubulin alpha-1B chain), cytoskeleton organisation (brain-specific angiogenesis inhibitor 1-associated protein 2, cofilin-1, F-actin-capping protein subunit alpha-1, phakinin, actin, cytoplasmic 1, actin, cytoplasmic 2), microtubule-based processes (tubulin alpha-1D chain, tubulin alpha-3 chain, tubulin alpha-4A chain, tubulin beta chain, tubulin beta-4B chain, tubulin beta-6 chain, tubulin beta-2B chain) or peroxisome biogenesis (peroxisomal membrane protein 11B). Expression of these proteins is, likely, the result of significant changes in mammary matrix and mammary epithelial cells, caused by virulence factors of the challenge organism, given that in previous studies of *M. haemolytica*-associated mastitis extensive lesions have been seen in the mammary parenchyma during histological examination (Mavrogianni et al. 2005, Fragkou et al. 2010). The modification of exposed sulfhydryl groups in cytoskeletal proteins may play a regulatory role, thus transducing oxidative stress signals into cytoskeletal changes (Wilson and González-Billault 2015). Tubulins are cysteine-rich redox-sensitive proteins implicated in cell division; modification of tubulin redox state or alkylation of functional sulfhydryl groups would lead to impairment of microtubule polymerisation and inhibition of cellular proliferation (Landino et al. 2004); in general, increase of these proteins may be related to their role in vesicular trafficking (Addis et al. 2013).

The 11 differentially expressed proteins involved in glycolytic processes, participated specifically in glycogen catabolism (glycogen phosphorylase, muscle form), in glycolipid biosynthesis (galactose-3-O-sulfotransferase 3, muscle form), in glycolysis (e.g., alpha-enolase), in mannose biosynthesis (phosphomannomutase 2) or in glyconeogenesis (triosephosphate isomerase). Alpha-enolase has been found to catalyse formation of phosphoenol-pyruvate from 2-phosphoglycerate, the product of phosphoglycerate kinase 1 (Verma and Dutta 1994), to serve as a receptor and activator of plasminogen on the surface of various types of cells, e.g., leucocytes, and to stimulate immunoglobulin production (Felez et al. 1996). Alpha-enolase and beta-enolase have been identified in gels at early stage of infection, obviously triggered by the bacterial invasion, and, likely, have participated in the regulation of the defence response of the mammary gland; progressively, they have disappeared, as they had accomplished their role. In general, the glycolytic processes aim to produce high-energy molecules, which would be employed by the animal to cover the increased energy needs, paramount to support cellular activities occurring during the bacterial invasion of the mammary gland, thus supporting and complementing the

defensive activities: leucocyte mobilisation, phagocytosis, intracellular killing (Díaz-Ramos et al. 2012).

Moreover, six proteins were involved in lactation. Their observed changes may be the consequence of increased detection of casein peptides following proteolysis (Saeman et al. 1988, Le Roux et al. 1995, Somers et al. 2003, Wedholm et al. 2008, Larsen et al. 2010), which can be a direct effect of bacterial action or a consequence of the increased presence of proteases, released by neutrophils or leaked from blood into milk (Hinz et al. 2012), as increased proteolytic activity of milk decreases casein concentrations (Le Roux et al. 1995), rather than of increased protein production. In fact, protein synthesis has been known to be reduced during bacterial infection (Danielsen et al. 2010). In previous proteomics studies, in cases of mastitis in cows, significant reductions in concentrations of caseins, beta-lactoglobulin and alpha-lactalbumin have been detected, present results being similar to those.

The six proteins involved in metabolic processes, specifically in carbohydrate metabolic process (e.g., carbonic anhydrase 3), cholesterol, lipid and sterol metabolism (e.g., apolipoprotein A-I), lipid metabolism (e.g., peroxiredoxin-6) or nucleotide metabolism (e.g., nucleoside diphosphate kinase B), participate in various functions. Among these, glutathione S-transferase P is involved in conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles and regulates cyclin-dependent kinase 5 activity (Sun, Chang et al. 2011), carbonic anhydrase 3 is responsible for reversible hydration of carbon dioxide (Badger and Price 1994) and neprilysin cleaves angiotensin-1, angiotensin-2 and angiotensin 1-9 (Rice et al. 2004) and induces proteolysis (Le Moual et al. 1994).

Six differentially expressed proteins involved in stress response were identified 12 hours after inoculation. Heat shock proteins have already been associated with development of mastitis, specifically as regulators of susceptibility to development of clinical mastitis (Huang, Lu et al. 2015), indicating a potential role of mammary epithelial cells in their production (Addis et al. 2011). Given their early appearance after infection, they have also been proposed for possible use in the detection of bacterial invasion into the mammary gland (Chiang et al. 2008).

Further, eight proteins were involved in transport of ions and other molecules, specifically in ion transport (e.g., ATP synthase subunit beta mitochondrial, serotransferrin), lipid transport (e.g., apolipoprotein A-IV) or protein transport (e.g., alpha-actinin-4). Presence of serotransferrin and serum albumin and transthyretin in milk has been discussed extensively above. Nevertheless, synthesis of serum albumin also in the mammary gland has been reported before and would have also contributed in increase of protein content after infection (Shamay et al. 2005).

Finally, three differentially expressed proteins were involved in transport of oxygen, all being haemoglobin subunits. Haemoglobin would likely have originated from extravasation or micro-haemorrhages in mammary vessels, done under the influence of virulent factors of the causal organism.

#### General appraisal

The entirety of proteomics findings indicates that affected ewes mounted a defence response, which had been regulated by many proteins and through various pathways. These were interdependent at various points. Under the effect of cytokines produced by the defence system of the host, neutrophils entered into the infected mammary gland. Their mobility and function in the gland was considered to have been regulated by proteins involved in cell communications. Neutrophil degranulation led to, among others, release of antimicrobial proteins, of which a significant one was cathelicidin-1. Proteases have also been released from neutrophils that had arrived into the affected gland, were responsible for lysis of milk proteins (indicated by decrease of milk proteins) and damage to mammary parenchyma (Mehrzad et al. 2005).

The cathelicidins and S100 family proteins had clearly established antimicrobial role and had been produced by neutrophils, although expression might also have taken place in mammary epithelial cells (Lutzow et al. 2008).

Other proteins identified with differential expression constitute part of the non-specific antibacterial system of the mammary gland. Lactotransferrin has antimicrobial and antiinflammatory properties and has already been reported with upregulation in mastitis (Hagiwara et al. 2003, Fetherston et al. 2006); nevertheless, in the present study, it has been identified to fluctuate after animal inoculation. Lactoperoxidase has been found with downregulation, although it is considered as a non-specific antibacterial protein. Their observed reduction in mammary secretion samples after challenge may reflect their reduced concentration, subsequently to their involvement in the antibacterial process.

Further, it may be postulated that ewes attempted to continue milk synthesis and production, as well as accommodating the increased needs for leucocytic activities. This can be indicated by the involvement of proteins in the glycolytic processes and in metabolic processes for lactose production by mammary epithelial cells not affected by bacteria during the disease. To note that lactose is used by the organ during bacterial invasion to stabilise equilibrium with blood, in attempting to re-establish normality in pH, osmolality and electroconductivity of mammary secretion; these parameters would be severely modified during bacterial infection, as early as 6 hours after that (Fragkou et al. 2010), but the host would attempt to rebalance them, in order to continue milk production. Various proteins (e.g., serum albumin) can be considered to participate

in this process. To note that, as per theory of energy partitioning in sheep (Baldwin and Sainz 1995, Sandberg et al. 2007), energy needs for reproduction (which includes lactation) are prioritised over needs for immunological response of animals; this indicates that, in terms of allocation of energy resources, continuation of milk production is of higher priority for a ewe than production of defence cells, despite that being a paradox for a ewe with mastitis.

Increased blood serum albumin in milk samples, coupled with increased somatic cell counts, indicated by higher California Mastitis Test scores, which are a good proxy for somatic cell counts in ewes' milk (Fthenakis 1995), point out to an influx of blood constituents into milk. Increased somatic cell counts and protein upregulation were both noted 12 h after bacterial deposition into the teat, which underlines their interactions.

Certainly, not all proteins observed with new expression or upregulation in milk were of blood origin. Many of the proteins in milk samples had been produced locally by mammary epithelial cells or released by neutrophils after their influx into the mammary gland. Indeed, apart from bacterial products, proteins released from neutrophils might also cause a damaging effect in mammary tissue and further expression of proteins by affected tissue, as described above for haptoglobin. In cows with mastitis, it has been suggested that plasmin was the primary source of proteolytic activity in mammary tissue and mammary secretion (Schaar and Funke 1986, Verdi and Barbano 1991, Fang and Sandholm 1995).

The findings confirm the complex interactions between mediators, leucocytes, mammary cells and lacteal secretions that take place during acute inflammatory reaction in mastitis. It is noteworthy that the study focused in early stage of infection, where limited presence of lymphocytes is involved. These cells possess properties and fulfil functions other than those described in this study, in which other proteins would be involved and results would be different than present ones. A hint for this was evident by the new expression of interleucine-4 (protein with a role in recruitment of lymphocytes) on the fourth day after challenge.

A notable finding in this study was the difference in various details from findings reported by other researchers in the same field. There are many reasons to account for these differences. First of all most relevant studies have been carried out in cows (*Bos taurus*), an animal species different than sheep (*Ovis aries*). Apart from that fundamental difference, the two animal species are farmed under different production systems and management conditions, which would further enhance innate differences between them. Even in sheep, differences in breeds studied in the various projects might also have led to varying results, as there is a confirmed breed susceptibility to mastitis in ewes (Fragkou, Skoufos et al. 2007, Rupp et al. 2009). These may be reflected in the expression of proteins, regulated by various genes. Different protocols employed in the

experimental design, as well as in methods used would have also contributed in the differences recorded between the present and previous relevant studies. Finally, different pathogens that had been the causal agents of mastitis, in experimental or clinical settings, would have led to differences in results, as *M. haemolytica*-associated mastitis had never before been studied by proteomics methodologies.

## **Potential use of cathelicidin-1 in the diagnosis of *Mannheimia haemolytica*-associated mastitis**

### **Diagnosis of mastitis in sheep**

In sheep, for effective treatment of mastitis, an early and accurate diagnosis of the disease is important. Bacteria can cause damage to mammary epithelial cells as early as 24 hours after bacterial invasion, hence treatment should start early, as soon as possible after that. Diagnosis of clinical mastitis is based on recognition of clinical signs, which may nevertheless take 24 to 36 hours to develop, a period during which bacteria would continue causing a damaging effect in mammary epithelial cells. In contrast, diagnosis of subclinical mastitis is based on a combination of methods (Fragkou et al. 2014).

A variety of methods and techniques is employed for diagnosis of the disease. In brief, these include clinical examination (Fthenakis 1994), ultrasonographic examination (Franz et al. 2001, Lazaridis et al. 2012), endoscopy (Kiossis et al. 2009), bacteriological examination of milk samples, immunoassays -especially in cases of systemic diseases affecting the mammary glands, e.g. brucellosis (Garin-Bastuji et al. 2006, Juste et al. 2013), leptospirosis (Dagleish et al. 2010, Malone et al. 2010), mycoplasmal infections (Nicholas 2000, Nicholas et al. 2008) or lentiviral infections (Villoria et al. 2013)- in blood or milk samples, cytological examination of milk samples (Berthelot et al. 2005) or measurement of electroconductivity in milk samples (Romero et al. 2012).

As mentioned above, clinical mastitis is diagnosed easily, by recognition of changes in milk and/or affected mammary gland. For diagnosis of subclinical mastitis, of the above, the best method is the combination of bacteriological and cytological examination (Fragkou et al. 2014). Nevertheless, there is still some debate regarding norms in the cytological examination of milk for detection of inflammatory reaction in the mammary gland of ewes. Specifically, no well-defined



and fully acceptable thresholds of somatic cell counts have been set, in a way similar to those existing for cows' milk. Further, a variety of factors (e.g., age of ewe, breed of ewe, stage of lactation, number of lactation, milk yield, time of the day at sampling, daily frequency of milking, number of lambs suckled) may influence results of somatic cell counting (Raynal-Ljutovac et al. 2007), hence affecting diagnosis; these, therefore, should be taken into account in making a diagnosis of subclinical mastitis.

Berthelot et al. (2005) have indicated a valuable approach regarding thresholds indicating infection. In individual animals, values  $<0.5 \times 10^6$  cells  $\text{mL}^{-1}$  indicate a healthy mammary gland and values  $>1.0 \times 10^6$  cells  $\text{mL}^{-1}$  indicate a mammary gland with mastitis, with no need to perform a bacteriological examination of milk samples below or above these thresholds to confirm the problem. Values between  $0.5 \times 10^6$  and  $1.0 \times 10^6$  cells  $\text{mL}^{-1}$  point out to suspicion of the disease, in which case it is necessary to perform bacteriological examination in milk. Accuracy of results can be increased by performing two sequential cell counts. To note that other authors have proposed different values, even as low as  $0.25 \times 10^6$  cells  $\text{mL}^{-1}$  (Ariznabarreta et al. 2002), to indicate the threshold defining mastitis; this indicates the lack of consensus regarding norms for somatic cell counts in ewes' milk. In order to improve diagnosis, identification of cell types in milk samples can be performed, given that milk of healthy ewes contains mainly macrophages, whilst during subclinical mastitis neutrophils (acute phase) or lymphocytes (chronic phase) predominate (Fthenakis and Jones 1990).

It is noteworthy, that somatic cell counting and evaluation of electroconductivity in milk samples are, in reality, techniques identifying biomarkers into the milk, measuring the former leucocytes and the latter ions present in milk (Fragkou et al. 2014). Somatic cells, i.e. leucocytes, enter into the mammary gland as part of the defensive action of the host. Ion concentrations in milk change as potassium ions leak out of the mammary gland, sodium ions from blood leak into the mammary secretion and chloride ion concentrations increase in mammary secretion during mastitis (Auldist et al. 1995, Auldist and Hubble 1998).

## Identification of proteins in milk as indicators of mastitis in sheep

In the past, various proteins have been discussed as having a potential significance in diagnosis of mastitis in cows. These included the chaperonins (taking part in pathogen recognition), various leucocyte-associated proteins (e.g., cathelicidin, peptidoglycan recognition protein, lymphocyte cytosolic protein 1, macrophage scavenger receptors) and various whey-proteins (e.g., beta-2-microglobulin, alpha-enolase, chitinase-3-like protein 1, inter- $\alpha$ -trypsin

inhibitor heavy chain H4, prostaglandin D synthase, S100-A12, serotransferrin, serum amyloid A, serpin A3-1, vitronectin) (Fragkou et al. 2014). Safi et al. (2013) have further reported differential milk protein expression patterns during mastitis caused by differing bacteria, which might be of help in the identification of the causative agents of the disease.

Cathelicidin proteins have been proposed to serve in identifying mastitis in the absence of clinical signs or as an indicator of the stage of infection (Lippolis and Reinhardt 2005, Boehmer et al. 2008, Smolenski et al. 2007, 2011, Pongthaisong et al. 2015, 2016). The proteins have also emerged as potentially reliable biomarkers in studies carried out in ewes with natural infections (Addis et al. 2011, 2013, Pisanu et al. 2015), findings that are supported by results of relevant studies of other authors in cows or goats (Murakami et al. 2005, Ibeagha-Awemu et al. 2010, Tomasinsig et al. 2010, Smolenski et al. 2011, Brenaut et al. 2014).

From the results of the first Experiment, as well as based upon observations of previous studies, cathelicidin-1 was singled out for further detailed investigation, particularly during the initial stage of mammary infection. In the second Experiment, a close monitoring of the initial stage of the disease was performed. Samples were collected from experimental animals at regular intervals for 24 hours after inoculation. One ewe was challenged with *M. haemolytica*, i.e., the same organism that was used in the first Experiment (for welfare reasons, no other ewe was included in the study, as the work, to some extent, replicated that performed in the first Experiment). Another two ewes were challenged with *S. chromogenes*, an organism with confirmed pathogenicity for the mammary gland (Fthenakis and Jones 1990), which was used to confirm the validity of identifying a non-specific biomarker, as is cathelicidin-1.

It has been observed that 3 h and 6 h after challenge, in 5 of 6 samples in total, cell counts in mammary secretion were between  $0.5 \times 10^6$  and  $1.0 \times 10^6$  cells in total, i.e., providing a suspicion for disease, although the animals had confirmed mastitis subsequently to intramammary inoculation of bacteria. Similar findings, indicating suspicion of the disease, were recorded in the scores of the California Mastitis Test (CMT). In this case, results of bacteriological examination, i.e., isolation of bacteria, were necessary to confirm the disease. In contrast, in 5 of the above 6 samples, cathelicidin-1 was identified. Further, the results confirmed presence of a strong correlation between somatic cell counts / CMT scores and identification of cathelicidin-1 in mammary secretion samples. The correlation was evident when either quantitative (numerical values) or qualitative (positive/negative) assessment was taken into account.

This correlation is reflected in the increased sensitivity of use of cathelicidin-1 identification with mastitis status. For definition of mastitis, the combination of bacterial isolation and increased somatic cell counts was used. Although in the present study, mastitis was induced experimentally,

i.e., status of the mammary gland was known, by use of this strict criterion it has become possible to simulate conditions in the field (Jones and Lanyon 1987), where the combination of bacteriological and cytological examination would be used for improved accuracy. It is noteworthy that when samples were received for processing by proteomics techniques, their precise origin was not known; this was revealed later and correlations were carried out. Performance of a blinded experiment increases value of results.

The results confirm the potential use of detection of cathelicidin-1 in the diagnosis of *M. haemolytica*-associated mastitis, which was part of the general remit of the present thesis. Nevertheless, it has been found that the approach may also be of use for diagnosis of mastitis caused by other organisms, as similar findings were evident after inoculation with *S. chromogenes*.

The greatest advantage of using cathelicidin-1 detection is the early diagnosis of the disease that may thus be achieved. Cathelicidin-1 is a neutrophil-associated protein, therefore presence in milk is dependent on influx of neutrophils into the mammary gland. The protein is released upon exposure of neutrophils to pathogens, which takes place as soon as these cells enter into the mammary gland at the early stage of infection. Likely, this is the reason that it provides a more accurate identification of mastitis at an early stage, i.e., when neutrophils had already entered into the mammary gland to counteract a bacterial invasion, but, yet, would not be at sufficiently increased numbers to indicate abnormal results in the cytological tests.

As cathelicidin-1 is not present in the milk of healthy ewes, there would be no need to establish a threshold, hence a 'positive'/'negative' assessment would suffice. On the other hand, this may lead to 'false positive' results yielded by presence of cathelicidin-1 in contralateral glands.

In the field, identification of cathelicidin-1 in pre-milking samples of milk would be particularly useful in contributing in detection of early stage mastitis, as its release soon after bacterial invasion would enable early detection of infection (Smolenski et al. 2011, Addis et al. 2013). As it has not been found to be associated with specific pathogens (as stated above, in the present study, it was detected after challenge with *M. haemolytica* or *S. chromogenes*; further, in previous studies, it has been detected during mastitis caused by other pathogens), it can be employed for detection of an infection independently of the causal agent. The fact that animals are routinely milked twice or thrice daily is further supporting its potential, because it can thus help in diagnosing an infection that had occurred in the previous 12 or 8 hours. This may be the most beneficial use of detection of cathelicidin-1 as a biomarker for diagnosis of mastitis.

Addis, Tedde, Dore et al. (2016) and Addis, Tedde, Puggioni et al. (2016) have described development of an ELISA to detect cathelicidins in milk. Perhaps, with further development, a

rapid test may be produced for animal-side use. This test would be based on this quick ELISA and would potentially be available for incorporation in milking systems, that way detecting the protein during machine-milking of animals in a farm. However, there is still need for further validation studies, before this development can be justified and sustained.

A setback for use of cathelicidin-1 as a potential marker for the disease was its detection in samples from the contralateral mammary gland. This was obviously the result of neutrophil influx into that gland, leading to release of cathelicidin-1 and its subsequent detection, although no increased leucocyte counts were recorded.

## Epilogue

### Conclusions

The conclusions from the results of the present thesis are summarised herebelow.

(a) The proteome of blood and milk of ewes with mastitis associated with *Mannheimia haemolytica* has been studied, for identification and evaluation of changes in protein expression, interactions or modifications as the result of mastitis. The findings confirmed the complex interactions between mediators, leucocytes, mammary cells and lacteal secretions that took place during acute inflammatory reaction in mastitis.

- The entirety of proteomics findings has indicated that affected ewes had mounted a defence response that had been regulated by many proteins and through various pathways; these were interdependent at various points.
- The results have indicated that already 12 hours subsequently to bacterial deposition into the teat duct, new expression and/or upregulation of increased number of proteins was evident, primarily in milk and to a lesser degree in blood.
- Of the proteins observed with new expression or upregulation in milk, some were of blood origin, some were released by blood constituents that entered into the mammary gland (e.g., by neutrophils) and some were locally synthesised.
- It may be postulated that ewes attempted to continue milk synthesis and production, as well as accommodating the increased needs for leucocytic activities.
- There were indications that various proteins might be of value as biomarkers for ovine mastitis.

(b) Detection of cathelicidin-1 in milk has been associated with increased sensitivity and specificity in diagnosis of mastitis.

- There was an increased correlation of presence of cathelicidin-1 with results of cytological examination; similar findings were recorded when cytological results were taken into account as either quantitative (numerical values) or qualitative (positive/negative) results.
- Cathelicidin-1 was detected in milk earlier than increased cellular content.
- When used for diagnosis of mastitis, cathelicidin-1 had the advantage that, as it is not present in milk of healthy ewes, there would be no need to establish a threshold, hence a 'positive'/'negative' assessment would suffice.

## Prospects

Suggestions for further research, in continuation of the present work, are as below.

- The detailed study for pathogenesis of mastitis, through the evaluation of protein interactions during the acute phase of the disease, by using new technological approaches.
- The proteomic analysis of subacute stage of mastitis (i.e., after 4th day after infection), especially of the role of lymphocytes.
- The evaluation of using cathelicidin-1 in routine diagnosis of mastitis at an early stage in farm conditions.

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